FREE RADICAL RELATED BIOCHEMICAL ALTERATIONS IN LOCOMOTOR AND RESPIRATORY MUSCLES FOLLOWING ACUTE EXERCISE IN YOUNG ADULT AND SENESCENT FEMALE FISCHER-344 RATS

By

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This work is dedicated to my wife Lynn Garrot Lawler for the patience, assistance, and, most of all, love she has provided me during the last three and one-half years of my life.

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Free radical toxicity has been implicated as contributory to both aging and skeletal muscle pathology. Indeed, recent evidence suggests that free radicals are produced primarily as function of oxidative metabolism, and that an acute bout of exercise results in an escalation of free radical formation in locomotor muscle resulting in disruption in sarcoplasmic reticulum and mitochondrial function. The diaphragm is the primary respiratory muscle and is essential to life. However, no reports exist concerning the effects of acute exercise on free radical related biochemistry in young or senescent animals. Thus, we sought to test the hypotheses that 1) the elevated antioxidant status of the diaphragm would protect it from metabolic enzyme damage as compared to locomotor muscle; and 2) acute exercise in senescent animals would exacerbate exercise-induced changes in skeletal muscle lipid peroxidation and metabolic enzyme activities. Twenty 4 month old and twenty 24 month old female Fischer-344 rats were acclimated to treadmill running; each age group was then divided at random into young exercised (YE); and old exercised (OE) and young control (YC); and old control (OC) groups. Animals in both young and old exercise groups ran on a rodent treadmill for 40 minutes at 75% estimated $\dot{V}O_2max$. Following the treadmill run, both exercise and control groups were euthanized with sodium pentobarbital. Costal (COSD) and crural diaphragm (CRU), plantaris (PL), white and red gastrocnemius (WG and RG), and soleus (SOL) muscles were removed and quickly frozen in liquid nitrogen. Results revealed a significant ($P<0.05$) increase in lipid peroxidation in YE COSD and RG. Activity of the antioxidant enzyme glutathione peroxidase was significantly elevated in YE and OE plantaris and YE white gastrocnemius. In addition, ANOVA revealed a significant overall main effect for increased antioxidant enzyme superoxide dismutase with exercise ($P<0.05$). Metabolic enzymes phosphofructokinase (PFK), citrate synthase (CS), and 3-hydroxyacyl-CoA dehydrogenase (HADH) were unchanged with acute exercise in the diaphragm. However, PFK was inhibited in the WG by 60% in OE and 33% in YE, and in the RG by 41% in OE. *In vivo* allosteric modifiers of PFK (i.e., $[H^+]$, disulfide redox state, [citrate], ADP, ATP, free fatty acids) could influence the magnitude of downregulation in working muscle. These data indicated an increase in exercise-induced free-radical production in YE and to a lesser extent OE; metabolic enzymes were well protected at this exercise intensity and duration in the diaphragm. It is probable that the greater degree of exercise-induced gastrocnemius PFK inhibition in the senescent animals was due to greater susceptibility of PFK in this age group to "downregulation." Future investigations are needed to ascertain the causality of free radical toxicity and muscle enzyme downregulation, quantification of contribution and location of free radical sources in working skeletal muscle, influences of exercise intensity and duration as well as motor unit recruitment pattern on free radical toxicity in working skeletal muscle.

INTRODUCTION

In eukaryote organisms molecular oxygen (O_2) is essential to cellular metabolism and life itself. Yet the very physical nature of chemical reactions involving molecular oxygen results in production of potentially dangerous oxygen intermediates and subsequent products (e.g., free radicals, nonradical oxygen intermediates, and hydroperoxides) which pose a great threat to cellular homeostasis. Free radicals are extremely reactive and are prone to initiating chemical chain reactions (39). Endogenous production of free radicals is thought to originate from the interaction of molecular oxygen with a host of substrates, catalysts, and oxyradicals themselves (64,94). Eukaryotes have developed two broad strategies for management of O_2 intermediates: a) controlled integration and utilization of free radicals into essential cellular and systemic function; and b) a multilayered molecular, cellular, and organ level antioxidant system designed to limit the concentration of free radicals and hydroperoxides, as well as repair of free radical-induced cellular damage. If the antioxidant defense system is overwhelmed, cellular pathology, aging, and cell death may occur (15,20,111,115). In fact, the relationship between production and propagation of free radicals (as well as their toxic products) and available antioxidant defense systems underlies the pathology of many disease processes (15), including those found in skeletal muscle. Recently, Franceschi et al. (39) have proposed that antioxidant defense systems are an integral part of an interconnected network of cellular defense mechanisms which minimize the effects of internal and external biochemical stressors. The proposed network of cellular defense mechanisms includes DNA repair, detoxification of xenobiotics, heat-shock response, and poly(ADP-ribosyl)ation.

A number of pathological conditions such as muscular dystrophy, ischemia/reperfusion injury, inflammation, aging, and possibly fatigue have been associated with free radical toxicity in skeletal muscle (64,66,68,86,110,113,127). Normally, at rest, the layered systemic and cellular antioxidant defense system of antioxidant enzymes and substrates (e.g., superoxide dismutase, catalase, glutathione peroxidase, Vitamin E) ameliorates the toxic effects of biologically generated oxyradical production. Mounting evidence suggests that free radical production is increased, especially through "metabolic leaks" in the electron transport chain, with acute exercise (26,30,57,64,68). Further, oxygen-derived free radicals have been implicated in elevation of lipid peroxidation (2,3,6,26,101,102) and a partial inactivation, or "downregulation," of metabolic enzymes (8,31,32,46,47,68) observed on occasions in locomotor skeletal muscle immediately following a bout of acute exercise. Extensive damage to lipid membranes via free radicals alters membrane fluidity, membrane potential, permeability of membranes to macromolecules, and alteration of membrane protein function (most importantly Na^+/K^+ ATPase and Ca^{2+} ATPase: 63,103,112). In addition, free radical biochemistry may play a role in mitochondrial and sarcolemmal disruption following strenuous exercise (14,46,47,68) as well as myofilament damage following eccentric exercise (5,18,128).

Although enhanced lipid peroxidation and antioxidant enzyme levels in resting locomotor muscle have been noted by Ji et al. (66) in aged rats when compared with young adult rats (indicating increased skeletal muscle free radical production with age), reports concerning free radical related biochemical alterations with acute exercise in senescent animals are limited. Ji et al. (66) reported significant increase in cytosolic glutathione perxoxidase activity (or $\dot{V}_{max}$) following 1 hour of level treadmill running at ~60% $\dot{V}O_2max$ in 24 and 31 month old male Wistar-Furth rats. Recently Wu et al. (126) demonstrated that the activity of glutathione peroxidase, but not catalase or superoxide dismutase, is increased by 10 weeks of treadmill endurance training in 24 month old female Fischer-344 rats.

There are no reports in the literature, in young or old animals, concerning acute exercise-induced free radical mediated lipid peroxidation and metabolic enzyme inactivation in the diaphragm. This is unfortunate considering that the mammalian diaphragm is the principal respiratory muscle and is the only skeletal muscle essential to life and that procurement of such information has important physiological and pathophysiological ramifications. Research on senescent animals is important as mitochondrial (53) and cellular aging (27,93) have been associated with chronic, repetitive free radical toxicity. The animal model lends itself for such study.

The major objective of the current investigation is to improve the understanding of free radical production in the diaphragm and its relation to acute submaximal exercise, and to examine any interactive effects of senscence. For comparative purposes, selected locomotor skeletal muscle differing in fiber type will also be studied (i.e., plantaris, red and white gastrocnemius, and soleus). The global hypothesis tested was that muscles which have a lower relative (per unit weight) oxygen utilization during submaximal exercise will be more susceptible to free radical related biochemical alterations. Further, an attempt to disprove that relative tissue oxygen flux alone is primarily responsible for free radical related damage in skeletal muscle was studied. The following specific hypotheses were tested:

1. Acute exercise results in less disturbance of cellular homeostasis as measured by lipid peroxidation and enzyme downregulation in the diaphragm as opposed to the plantaris; the augmented antioxidant status of the diaphragm provides greater protection against free radical related biochemical alterations than locomotor muscles.

2. Respiratory and locomotor muscles in senescent Fischer-344 rats are more susceptible to free radical-related metabolic enzyme disturbances and lipid peroxidation than young rats.

3. Enzymatic downregulation with a bout of acute exercise, determined via biochemical assessment of important metabolic enzyme activities, will be less prevalent in type IIb-fast twitch glycolytic muscle (white gastrocnemius) than type IIa-fast oxidative glycolytic (red gastrocnemius) or type I-slow twitch (soleus) muscle since fast glycolytic muscles are recruited less during moderate submaximal work.

Acute exercise induced free radical-related biochemical alterations in skeletal muscle were determined via assessment of "downregulation" or inactivation of metabolic enzyme activities, changes in antioxidant enzyme activities, and increases in lipid peroxidation. The metabolic enzyme markers phosphofructokinase, citrate synthase, and 3-hydroxyacyl-CoA-dehydrogenase were chosen because of their potential rate-limiting state in glycolysis, citric acid cycle, and ß-oxidation (lipid oxidation), respectively. It follows that sizable inactivation in these enzymes would diminish the effectiveness of the corresponding metabolic pathways to generate ATP. Total tissue glutathione peroxidase and superoxide dismutase were chosen as markers of antioxidant enzyme status. Measurement of muscle malondialdehyde levels was used to estimate lipid peroxidation.

REVIEW OF LITERATURE

The general purpose of this section is to provide a review of current literature concerning free radical biochemistry in skeletal muscle with an emphasis on acute exercise. The review is separated into three divisions. The first section discusses free radical production and management in skeletal muscle. The focus of the second section is upon skeletal muscle tissue injury and relation to free radical toxicity. The final portion of the review concentrates on aging, the diaphragm, and free radical biochemistry.

Free Radical Biochemistry and Skeletal Muscle

Molecular oxygen (O_2) as well as oxygen intermediates can be perceived as possessing a "Janus" biochemistry: sources of tremendous metabolic, anabolic, and destructive power. Oxygen-derived free radicals participate in many normal cellular and organ functions as well as contribute to the pathology of a large number of disease states. Free radicals have been hypothesized as participating in numerous important, normal cellular functions such as phagocytosis of microorganisms, detoxification of xenobiotic compounds, alcohol oxidation, vasodilation, protein activation and turnover, glycogen repletion, prostaglandin synthesis, and biogenesis of mitochondria or whole cells (4,17,22,24,64,71,84,87). However, free radical toxicity can pathologically alter structure and function of proteins, lipids, carbohydrates, and nucleic acids. Disturbances in cellular homeostasis and function resulting from free radical toxicity have been implicated as contributing to the pathology of over one-hundred syndromes and disease processes which include cancer, artherosclerosis, stroke, arthritis, cataracts, diabetes, emphysema, adult respiratory distress syndrome, and aging (52,53,61,64,93). Further, biological free radicals have been demonstrated in skeletal muscle to be integral in muscular dystrophy, delayed-onset muscle soreness, inflammation, as well as ischemia/reperfusion injury (5,64,70,112,113,128). A limited number of reports suggest that free radical toxicity may play a role in muscle fatigue (63,86,107) as well. Therefore, comprehension of endogenous free radical production and management of biological free radicals is vital to our knowledge of skeletal muscle physiology and pathophysiology.

Free Radical Production

In general, the current knowledge of the effects of free radical toxicity on skeletal muscle, with its physiological and therapeutic implications, lags well behind that found in more well-described tissues such as the liver and the lung. Thus, a brief review of free radical biochemistry with emphasis on production in skeletal muscle follows. Atoms in most chemical compounds are bonded in a covalent manner possess electron pairs in the outer orbitals which have opposite spins (of +1/2 and -1/2), as dictated by the Pauli principle (51,110). The term "free radical" refers to a molecule which possesses an unpaired electron in the valence shell (64); such compounds include hydrogen atoms, most transition metals, and molecular oxygen (52). Molecular oxygen (O_2), or dioxygen, is potentially susceptible to radical formation as it is in actuality a stable "diradical:" that is, O_2 has two unpaired electrons in its outer orbital shell. Most elements and the majority of organic compounds are potentially susceptible to oxidation by dioxygen (57).

Fortunately, kinetic restrictions exist concerning the reactivity of molecular oxygen in its ground state (i.e., $^3\Sigma gO_2$) (48,57). The two unpaired electrons have parallel spin (90) and are located in different outer orbitals (40) which greatly diminish the spontaneous reactivity of molecular oxygen ($^3\Sigma gO_2$) as dictated by quantum mechanics. Therefore, in order for dioxygen to react rapidly, the spin restriction must be removed, the O--O bond weakened, or both. However, dioxygen reactions with single electrons, hydrogen atoms, or other species containing unpaired electrons (i.e., most transition metals) can overcome those restriction. Most transition metals, especially important are Fe^{2+} and Cu^{2+}, facilitate the single electron transfer with molecular oxygen (115). Finally it is noteworthy that free radicals can act biochemically as both strong oxidants or reducing agents (115).

Singlet oxygen

The conversion of molecular oxygen to its excited singlet electron state ($^1\Delta gO_2$ or $^1\Sigma g^+O_2$) via radiant, chemical, or thermal energy removes the spin restriction (57) and weakens the O--O bond thus increasing the reactivity. However, the chemical importance of the $^1\Sigma g^+O_2$ state is small as it decay very rapidly into $^1\Delta gO_2$. The $^1\Delta gO_2$ state, which is not a radical but is reactive, may be produced when biological pigments such as retinal, flavins, porphoryns, and chlorophyll are exposed to light with sufficient O_2 available (52). Estimates of the lifetime of singlet dioxygen are 4 µsec in aqueous solutions and 25-100 µsec in nonpolar organic media similar to membrane environs (38). Major susceptible cellular targets for $^1\Delta gO_2$ oxidation are lipid membranes; guanine residues of nucleic acids; and histidine, methionine, tryptophan, tyrosine, and cysteine residues of proteins and enzymes (38). Tissues exposed to radiation or UV light such as the lens and retina of the eye, as well as the skin, are subjected to significant chemical insult from singlet oxygen production (59). The degree of $^1\Delta gO_2$ production in skeletal muscle is poorly understood but is likely to be integrated with lipid hydroperoxide production. Catalase metabolism of hydrogen peroxide (H_2O_2) results in a small (0.5% of the total) release of $^1\Delta gO_2$ (17).

Superoxide anion

The primary oxygen intermediate biologically produced in mammalian cells is the superoxide anion ($O_2^{\bullet -}$). Superoxide anions may initiate cellular damage or act as precursors to other oxygen intermediates and peroxides. Cellular production of superoxide anions occur predominantly in the mitochondrial, microsomal, and peroxisomal compartments (17). In general, superoxide anions are formed via a process known as redox cycling, whereby electronegative compounds accept electrons and then reduce oxygen. Superoxide anions can be generated by a large number of enzyme, substrate and oxidase systems as well as autoxidizable small molecules and proteins (52).

The majority of free radical production in skeletal muscle is thought to be mitochondrial in origin and a function of metabolism (64). It is believed that approximately 2% of the oxygen consumption through the electron transport chain results in liberation of free radicals and peroxides (17), initially as superoxide anion ($O_2^{\bullet -}$). Indeed, Boveris et al. (9) demonstrated that ubisemiquinone is the major source of $O_2^{\bullet -}$ in the inner mitochondrial membrane. The sites of electron transport chain release of free radicals are believed to be NADH-coenzyme Q reductase (complex I or NADH-dehydrogenase complex) and ubiquinol-cytochrome c reductase (complex III) (9,16,64). Boveris and Chance (10) have shown that ubiquinone (also referred to as coenzyme Q or CoQ) related production of $O_2^{\bullet -}$ is related to ubiquinone reduction state and oxygen consumption. The ubisemiquinone reaction (18) appears to be preferred:

$$Q_{10}H^{*} + O_2 \longrightarrow O_2^{\bullet -} + Q_{10} + H^{+}$$

where $Q_{10}H^{*}$ is a reduced ubiquinone. Ubiquinones are non-protein, lipid-phase, highly mobile molecules which can move along the inner mitochondrial membrane between the flavin and cytochrome shuttle proteins (17). The release of $O_2^{\bullet -}$, via the electron transport chain or "metabolic leak" is directly related *in vitro* to the partial pressure of ambient oxygen (60,85).

However, the degree of "metabolic leak" mentioned above is true (~2%) for state 4 mitochondrial respiration (17), or the model for mitochondria in a "resting" muscle. The degree of "metabolic leak" in state 3 (activated; not ADP limited) respiration in mitochondria is unknown. Working skeletal mitochondria are in state 3 respiration control during exercise (25). As flux through mitochondrial electron transport chains increases dramatically during exercise, the production of $O_2^{\bullet -}$ via metabolic leaks should increase. As ubiquinones become more reduced with exercise, the amount of metabolic leaks could be exacerbated with exercise (25). Indeed, Jackson et al. (58) reported increases in electron spin resonance (ESR) (free radical signals) measured at -180°C immediately following 30 minutes of rat hindlimb *in situ* contractile work as compared with rest-controls.

It is important to note that cytochrome oxidase, which reduces oxygen to water and is the terminal sink for reactions in the electron transport chain, does not release free radical intermediates into the cellular milieu; they are tightly bound within the heme subunits (17) until reaction with molecular oxygen is completed:

$$O_2 + 4H^+ + 4e^+ \longrightarrow 2H_2O$$

Thus, oxyradical production may not be completely a "necessary evil" in utilizing molecular oxygen to produce cellular ATP. It is postulated that free radical signals may be involved in a metabolic control scheme not yet understood. Recently, K. Mailer (79) found that xanthine/xanthine oxidase generated $O_2^{\bullet-}$ (a powerful reductant under nonaqueous conditions) reduces cytochrome c *in situ* and is a source of electrons for oxidative phosphorylation. The physiological significance of this phenomenon remains to be determined.

It has been proposed that in skeletal muscle an important source of $O_2^{\bullet-}$ production is xanthine oxidase (110). This is at first perplexing as skeletal muscle itself has very low levels of xanthine oxidase (99,112). Studies by Jarasch et al. (61,62) using immunolocalization methodology reveal that xanthine oxidase (XO) is primarily located in the microvascular endothelium. This has been substantiated in isolated endothelial cells by Raytch et al. (96). In nonischemic tissue, NAD^+-xanthine dehydrogenase (XDH) is predominant (90,110,112) and does not produce $O_2^{\bullet-}$. Following ischemia-reperfusion, a significant portion of xanthine dehydrogenase is converted to xanthine oxidase (76), increasing production of $O_2^{\bullet-}$ and contributing substantially to cellular injury (83). Ischemia is thought to promote conversion of XDH —> XO through two mechanisms: a) inactivation of a cellular protease inhibitor which, under non-ischemic conditions, normally prevents a Ca^{2+}-activated protease from converting xanthine dehydrogenase into xanthine oxidase (99), and b) oxidation of sulfhydryl groups (28,90).

During exercise large quantities of hypoxanthine, xanthine, and urate (substrates and products) for both XDH and XO efflux from working muscle as indicated by increased plasma metabolite levels (54,55,110) and elevated arterio-venous differences (69). Conversion of xanthine to urate in working muscle, as inferred during exercise from plasma [urate] and net urate efflux from muscle, increases in a workrate dependent manner with a pattern similar to that of lactate and ammonia (69). Xanthine dehydrogenase/xanthine oxidase represents the terminal enzyme in adenine and purine nucleotide degradation cascade (69,100). Production of adenine nucleotide degradation metabolites via AMP deaminase (with concomitant ammonia production) is inversely related to the oxidative capacity of skeletal muscle (122). In other words, white muscle degrades larger quantities of adenine nucleotides, with therefore greater potential for substrate flux through XDH/XO, than red muscle. Degradation and depletion of adenine nucleotides increases in skeletal muscle under the following conditions: a) high intensity exercise, b) hypoxia or ischemia, c) during ß-adrenergic blockade, d) after detraining, and e) at low glycogen levels (100). Although xanthine dehydrogenase to xanthine oxidase conversion occurs with ischemia/reperfusion and hypoxia (28,99), such conversion has not yet been identified in skeletal muscle subject to acute exercise. Therefore, although it is apparent that an increase in XDH/XO substrate flux and release from working muscle (especially in white muscle or Type IIb) occurs with exercise, contribution of exercise-induced $O_2^{\bullet -}$ production via xanthine oxidase remains unknown.

Further, endothelial generated $O_2^{\bullet -}$ via xanthine oxidase can trigger chemotaxis mechanisms for polymorphonuclear leukocytes (96) and phagocyte activation, and thus amplification of cellular injury with ischemia/reperfusion (127). Activated blood-borne leukocytes then generate $O_2^{\bullet -}$ via NADH oxidase (11) or may release neutrophilic proteases (113), thus exacerbating tissue injury. In addition, activated leukocytes may release hypochlorous acid (HOCl), which can contribute to tissue injury (19). Indeed, etiology of ischemia/reperfusion injury has been suggested to be independent of intrinsic antioxidant status (119).

In addition to mechanisms mentioned above, other electron transport chain components (NADH dehydrogenase, iron-sulfur flavoproteins, and cytochrome b) cytochrome P450, numerous autoxidizable substrates and proteins, as well as NADH oxidase, glucose oxidase, and other oxidases all can contribute to the cellular generation of superoxide anion (16) albeit less effectively. Although not highly toxic in its nonpronated state (94), superoxide anion is directly toxic when pronated (hydroperoxyl radical), or chelated with a transition metal (15,17). Nonpronated superoxide anion functions as a reducing agent, capable of reacting with many compounds, and can cross membranes through anion channels (37). The pronation of superoxide anion has a pKa = 4.8; thus superoxide would be dissociated in the muscle cell interior (pH~7.0):

$$O_2^{\bullet-} + H^+ \Leftrightarrow HOO\cdot$$

However the pH of the environ in close proximity to cell membranes approaches that of the pK for pronation of superoxide anion, a result of strong ion differences across cell membranes. The half-life of $O_2^{\bullet-}$ is dependent on superoxide dismutase (SOD) activity and all substrates which react with superoxide anion (94). Thus, the half-life of $O_2^{\bullet-}$ would be longer in skeletal muscle than in liver or heart which both possess higher SOD activities. The toxic potential of superoxide anion is, to a large degree, dependent on the ability of $O_2^{\circ-}$ to form hydrogen peroxide (H_2O_2) and subsequently the highly toxic hydroxyl radical ($\cdot OH$) as discussed below.

Hydrogen peroxide and hydroxyl radical

Aqueous superoxide anion may undergo dismutation to form hydrogen peroxide (H_2O_2) and O_2; this reaction can occur spontaneously or be catalyzed by superoxide dismutase (17,94,115):

$$O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \Leftrightarrow H_2O_2 + O_2$$

The pKa of the dismutation of superoxide anion is very high (52); therefore, under physiological conditions the reaction is nonequilibrium. Chance et al. (17) have noted the cellular and systemic importance of superoxide dismutase in accelerating the above reaction, maintaining superoxide anion at a low level. Hydrogen peroxide itself is not very reactive, nor is it a free radical, and thus is capable of diffusing appreciable distances from site of production (94) and across cell and organelle membranes. This allows potential propagation of free-radical signals. Hydrogen peroxide will readily react with superoxide anion in the presence of ferrous or cuprous ions to form the hydroxyl radical.

Hydroxyl radical (•OH), an extremely toxic free radical intermediate, may be formed in biological systems by superoxide anion and hydrogen peroxide via the Haber-Weiss reaction (catalyzed by Fe^{2+}, Cu^{+}), or by hydrogen peroxide and Fe^{2+} via the Fenton reaction (17,52,115) as listed below:

Haber-Weiss: $O_2^{\bullet -} + H_2O_2$ --------> $O_2 + OH^- + \bullet OH$

Fenton: $Fe^{2+} + H_2O_2$ --------> $Fe^{3+} + OH^- + \bullet OH$

The hydroxyl radical generally causes cellular damage with virtually any organic molecule near the site of formation and has a half-life of 10^{-9} sec (94). Three types of reactions involving •OH are probable: a) hydrogen atom abstraction, b) addition (e.g., adding on an aromatic ring structure such as purine and pyrimidine bases of DNA), and c) electron transfer (52). The hydroxyl radical is thought to be responsible for much of the direct free radical damage in organic systems, as it has extremely high rate constants with virtually every cellular constituent including sugars, DNA bases, organic acids, phospholipids, and amino acids (52,115). The hydroxyl radical may initiate cellular pathology directly or act through genesis of lipid peroxidation (115).

Lipid peroxidation

Lipid peroxidation usually involves oxidation of organelle and plasma membrane lipid constituents. Extensive lipid peroxidation is capable of eliciting deleterious alterations in organelle and cellular homeostasis. Free radicals are highly reactive with polyunsaturated fatty acids (PUFA) such as those found in cellular membranes. In addition, Fe^{2+} and Fe^{3+} can contribute to initiation of lipid peroxidation (52). The lipid peroxidation process has three phases: initiation, propagation, and termination.

Lipid peroxidation is initiated when an organic free radical is formed via hydrogen abstraction of an unsaturated lipid (15,17,52). Such attack may be perpetrated by reactive species such as hydroxyl radical, molecular oxygen, singlet oxygen, protonated or chelated superoxide anion, hydrogen peroxide, ferryl hydroxy complexes, etc. (15,17,52). The illustrative example below involves attack by hydroxyl radical with formation of an organic or lipid radical:

$$\text{Lipid-H} + {\bullet}\text{OH} \longrightarrow \text{Lipid}^{\bullet} + H_2O$$

Following diene conjugation (stabilization), the organic radical reacts with O_2 to form a hydroperoxyl (peroxyl) radical (15,17,52):

$$\text{Lipid}^{\bullet} + O_2 \longrightarrow \text{Lipid-}O_2^{\bullet}$$

The peroxyl radical abstracts a hydrogen from an adjacent lipid forming a lipid hydroperoxide as well as a lipid radical:

$$\text{Lipid-}O_2^{\bullet} + \text{Lipid-H} \longrightarrow \text{Lipid-}O_2H + \text{Lipid}^{\bullet}$$

Now propagation of a chain reaction of lipid peroxidation (52) is likely. In addition, alkoxyl [RO•] (where R is a lipid)

$$\text{Lipid-}O_2H + Fe^{2+}\text{ complex} \longrightarrow Fe^{3+}\text{ complex} + OH^{-} + \text{Lipid-}O^{\bullet}$$

and peroxyl [ROO•] radicals

$$\text{Lipid-}O_2H + Fe^{3+}\text{ complex} \longrightarrow \text{Lipid-}O_2^{\bullet} + H^{+} + Fe^{2+}\text{ complex}$$

can be formed (52) from hydroperoxides in the presence of transition metal-salts (e.g., Fe^{2+}). These radicals (alkoxyl and peroxyl) further stimulate lipid peroxidation by abstracting hydrogens from other lipid molecules. Further, O_2 or HOO• can react with lipid hydroperoxides in generating alkoxyl radicals. In general, alkoxyl radicals are more reactive (18); and Fe^{2+} is considered more important than Fe^{3+} in lipid peroxidation. Additional lipid peroxidation products include aldehydes (including malondialdehyde) alkanes (i.e., ethane, pentane), epoxy-fatty acids, etc. (6,111). Extensive lipid peroxidation can adversely alter membrane fluidity, membrane protein function, ion conductance, Ca^{2+} homeostasis, increases in macromolecule permeability, and membrane potential (15,64,115). Further lipid peroxidation interacts with phospholipase A_2 activity which enhances lipid destruction (104); however, removal of damaged lipid membrane sections via phospholipase A_2 acts as a protective mechanism as well. In other words, cellular and organelle (i.e., mitochondrial) membrane function can be pathologically altered by excessive lipid peroxidation.

Termination of lipid peroxidation occurs when a lipid radical reacts with another lipid radical or is "quenched" by a free radical chain-breaking scavenger. The two most important lipid-soluble chain-breaking scavengers are œ-tocopherol (Vitamin E) and ß-carotene.

Oxygen Intermediate Management

Oxyradical production and damage are reduced and quenched by systemic, cellular, and repair systems. The systemic vascular system provides a number of antioxidant strategies for skeletal muscle and other body tissues. First, muscle blood flow provides a steady and sufficient supply of oxygen while maintaining low capillary and tissue PO_2. In addition, numerous extracellular proteins act as antioxidants predominantly by binding and inactivating transition metal stores. The liver provides antioxidant substrates during exercise, augmenting the systemic free radical protection. Cellular oxygen intermediate management consists of a) antioxidant enzymes (superoxide dismutase, catalase, glutathione peroxidase, etc.), b) free radical scavengers (œ-tocopherol, ß-carotene, reduced glutathione, ascorbate, urate, etc.), and c) free radical damage repair systems (protein degradation/inactivation, DNA repair, etc.).

Systemic antioxidant systems

The systemic vascular system is designed in order to deliver oxygen to the tissues at low but adequate tissue PO_2 levels (125). In addition, great care is taken to tightly bind the oxygen and transition metal (i.e., iron and copper) ions in the blood. Blood hemoglobin maximizes blood oxygen content and minimizes free or unbound oxygen. Similarly, blood iron stores are bound by hemoglobin and transferrin. When fully saturated, transferrin binds 2 mol Fe^{3+} per mole protein; however, transferrin is normally <50% saturated in the blood, and requires an anion such as bicarbonate for normal function (50). The plasma protein ceruloplasmin not only binds copper ions but also converts Fe^{2+} to Fe^{3+}, which is less likely to promote free radical production. Transferrin along with ceruloplasmin have been demonstrated as effective in preventing tissue lipid autoxidation *in vitro* (118). In addition, lactoferrin, which is secreted by neutrophils (50), has iron binding capabilities similar to transferrin. The plasma protein albumin also binds copper and effectively absorbs any redox cycling initiated by copper. In addition, histidine-rich glycoproteins, mucins, haptoglobins, and hemopexins all have transition metal-binding capabilities, thus minimizing oxidative damage (50).

Extracellular antioxidant enzymes and substrates also appear to have important local and systemic function in preventing systemic and tissue specific free radical toxicity. Extracellular superoxide dismutase (EC-SOD), proposed to be located in the vascular endothelium, provides protection against superoxide anion (80). This protection may be important following periods of local ischemia and infiltration by activated leukocytes. Vitamin E is transported in the serum by lipoproteins and potentially could provide additional oxidant protection. However, this protective effect appears to be small (50); in fact, lipoproteins may act as an electron acceptor and provide protection for Vitamin E (124) against free radical damage.

Erythrocytes may act as a free radical "sink" (50), since RBCs have high levels of the antioxidant enzymes superoxide dismutase, catalase, and glutathione peroxidase as well as free radical scavenger reduced-glutathione (GSH). High levels of antioxidant enzymes is, at least in part, a necessary protection for erythrocytes during exercise. During periods of increased O_2 oxygenation and deoxygenation in the blood, such as exercise, the likelihood of methemoglobin formation is higher (45); the formation of methemoglobin results in the production of superoxide anion. During intense exercise, high blood lactate levels may act as a buffer against this process with concomitant increased levels of blood $[H^+]$ reducing methemoglobin (42,45).

In addition, the liver provides some systemic antioxidant protection during exercise. The liver releases a substantial amount of reduced glutathione (GSH) during exercise (78). Lew et al. (78) report an increased level of plasma glutathione levels after acute exhaustion (90-120 min treadmill running) in the rat, which they attribute to a dumping of reduced glutathione from the liver. In addition, Gohil et al. (45) have found in humans an increased plasma total glutathione and GSH above resting levels 1-4 days post-exercise following 90 minutes of cycling at 65% $\dot{V}O_2max$. Further, Gohil et al. (45) reported a decreased reduced and total glutathione levels in the liver with acute exercise. Lew et al. (78) hypothesized that the mechanism for liver GSH release is related to increased plasma vasopressin levels during exercise. Hepatic release of GSH would appear to be a necessary antioxidant reserve mechanism as the ratio of reduced/oxidized glutathione in both working skeletal muscle and blood decreases with acute exercise (78).

Cellular antioxidant systems

A layered cellular system of antioxidant enzymes and substrates effectively ameliorates the toxic effects of biologically generated oxyradical production (64). Myoglobin binds cellular oxygen, acts as an O_2 buffer, and is thought to be involved in bound transfer of molecular oxygen to cytochrome oxidase (41). The cellular strategy of myoglobin then is to transport oxygen in the muscle cell to the mitochondria, to provide a low but fairly even PO_2 across the muscle cell, and to keep O_2 molecules in a bound state. Therefore, in skeletal muscle large changes in O_2 flux are accomplished with minimal alterations in sarcoplasmic PO_2 (125). Thus, tight control of cellular O_2 levels is an effective prophylactic measure against free radical generation. In addition, the cellular iron-storage protein, ferritin, minimizes free Fe^{2+} levels in the muscle cell. Ferritin has an amazing ability to bind iron: up to 4500 ions per molecule (52). However, if ferritin molecules are disrupted and iron stores are released, the potential deleterious impact on free radical management is obvious.

Antioxidant enzymes also provide considerable preventive protection against free radical toxicity. Superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, and glutathione-S-transferases provide an integrated antioxidant enzyme system in prevention of tissue damage.

Superoxide dismutase (SOD) catalyzes the dismutation of superoxide anion into hydrogen peroxide and molecular oxygen (17,64):

$$O_2^{\bullet -} + O_2^{\bullet -} + 2H^+ \longrightarrow H_2O_2 + O_2$$

Superoxide dismutase is found in both the mitochondrial (Mn^{2+}-dependent) and cytosolic (Cu^{2+}/Zn^{2+}-dependent) cellular compartments (67), and is effective in maintaining low superoxide anion levels in tissues (17). Mammalian Mn-SOD and Cu/Zn-SOD have dissimilar protein structures and teleological origins. Mammalian mitochondrial SOD is similar in structure to that found in prokaryotes. This supports the notion that the mitochondria, with its own genome, is a "captured" prokaryote organelle. Maintenance of a low cellular superoxide anion concentration appears to be vital for normal function (17). In fact, the ratio of SOD activity/metabolic rate is directly related to rodent and primate species lifespan (120). Maintenance of low $O_2^{\bullet -}$ levels by SOD protects catalase integrity.

Catalase (CAT) is confined to the peroxisomes and catalyzes reduction of H_2O_2 to water and O_2 (17).

$$2H_2O_2 \longrightarrow 2H_2O + O_2$$

Catalase can display peroxidatic function as well. Due to the compartmentalization of catalase and lower K_m when compared to GPX, this antioxidant enzyme may have somewhat limited effectiveness in skeletal muscle. However, the action of catalase protects superoxide dismutase integrity, thus completing a symbiotic relationship between the two antioxidant enzymes (19).

Glutathione peroxidase (GPX) is found in both the mitochondria and cytosol and reduces both hydrogen peroxide and hydroperoxide using reduced glutathione (GSH) as a hydrogen atom donor forming an alcohol, water, and oxidized glutathione (GSSG). It is now believed that glutathione peroxidase function is accomplished by family of enzymes: a selenium-dependent enzyme acts on both aqueous hydrogen peroxide and hydroperoxides; and glutathione-S-transferases catalyze the reduction of solely aqueous hydroperoxides (19,30,65,123). In addition, a phospholipid hydroperoxide Se^{++}-dependent glutathione peroxidase has been identified by Ursini and colleagues (123).

$$ROOH + 2GSH \longrightarrow ROH + H_2O + GSSG$$

Glutathione peroxidase is coupled with glutathione reductase in order to replenish GSH from the oxidized form of glutathione (GSSG). NADPH provides reducing power for the glutathione reductase reaction. NADPH, in turn, is produced in skeletal muscle mitochondria by $NADP^+$-specific isocitrate dehydrogenase and malic enzyme, and in the cytosol by the aforementioned enzymes and glucose-6-phosphate dehydrogenase. In skeletal muscle, GSH/GSSG ratios are maintained at a high level (~500:1) at rest providing a substantial reserve stock of antioxidant substrate (17).

The remaining important biological antioxidant substrates or "scavengers" function as lipid peroxidation "chain breakers" (111) and free radical quenchers. These can be divided into lipid soluble (œ-tocopherol, ß-carotene) and water soluble (ascorbate, reduced glutathione, urate) categories. Lipid soluble scavengers are incorporated into cellular membranes; œ-tocopherol is the most important (64,110). ß-carotene normally plays a minor role as a lipid peroxidation chain-breaker; however this function is enhanced at low tissue PO_2 levels (111) such as found in working muscle during exercise. Reduced glutathione (GSH) is a substrate for glutathione peroxidase as well as a lipid peroxidation chain-breaker. The major role of GSH may be in protecting protein sulfhydryl (-SH) groups associated with cysteine and methionine amino acid residues. Glutathione is synthesized from three amino acids: glutamate, glycine, and cysteine (29). The cysteine residue contains a sulfhydryl (thiol) group which is the site of action. The primary function of ascorbate (Vitamin C) in skeletal muscle is to repair œ-tocopherol (115); it can also function as a lipid peroxidation chain breaker and free radical scavenger, as well, but to a minor degree. Urate reduces hydroxyl radical as well as terminating lipid peroxidation. However, urate's function is limited as it is not lipid soluble. A number of other endogenous substrates such as glucose, malate, succinate, pyruvate, retinoic acid, CoQ, and cysteine possess antioxidant properties.

Protein degradation and nucleic acid repair

It is now apparent that proteolytic degradation of inactivated or damaged cellular proteins is yet another layer of the antioxidant defense system. It is well established that protein degradation increases with acute exercise (33,68); a portion of protein breakdown is attributable to use of amino acids as a source of fuel during exercise (4-10% of total fuel used). Proteins damaged by oxidative stress during exercise must be disposed of quickly and efficiently. Damaged proteins lose their 3-dimensional structure and form unwanted cross-links with other damaged proteins (89). Oxygen intermediates and hydroperoxides can alter primary, secondary, and tertiary structure of proteins (23). Insoluble aggregates of numerous cross-linked proteins may form exacerbating cellular damage (23,89). Protein degradation systems appear to minimize not only the effects of oxidative stress but also to take advantage of those processes. Proteolytic (proteinases and peptidases) systems degrade denatured/hydrophobic proteins damaged by oxidative stress into amino acid constituents much more quickly and more efficiently (89) than undamaged native proteins. This is especially important as protein sulfhydryl groups are susceptible to oxidation with acute exercise (68).

Different types of myopathy (disease or exercise-induced) may be identified by the proteins preferentially degradated and the proteases utilized. The type of protease degradation system used is believed to be related to the half-life of the protein, which in turn is related to protein structure (97). For example, specific thiol proteases degrade myofibrillar proteins (half-life of 41-200 hr) *in vitro* (97,106). Calcium-activated neutral protease (CANP) degrades heavy chain myosin, troponin, tropomyosin and œ-actinin. Cathepsin B and H are lysosomal proteases and degrade heavy chain myosin, troponin C, and tropomyosin; and troponin T, respectively. These systems are activated with acute exercise but are not inducible following 4 weeks of endurance training in rats (106). The activity of CANP increases with age, whereas the activities of the cathepsins appear to decrease with aging (106). Damaged amino acids residues, identity of the -NH_2 terminal amino acid in a polypeptide, and certain amino acid sequences within a protein are signals for degradation to commence (89,97). Histidine, cysteine, tryptophan, tyrosine, and methionine amino acid residues are susceptible to free radical damage.

DNA and RNA are susceptible to free radical attack. Lipid peroxidation metabolites such as malondialdehyde are especially destructive to nucleic acids. Thus, DNA and RNA repair systems may provide a secondary free radical defense system (23). A recent study by Alessio and Cutler (1) found increased urine levels of the DNA damage marker 8-hydroxydeoxyguanosine in humans 10 hours following a marathon race, and a subsequent elevation in DNA repair cycle activity. Further, free radical release from activated neutrophils may cause chromosome damage (52). In general, free radical damage of nucleic acids may involve mechanisms such as base hydroxylation, cross-linkage, and scission of DNA strands (115) resulting in mutations and inhibition of protein and fatty acid synthesis. Thus, nucleotide repair systems must comprise an important function in management of reactive oxygen intermediates.

Inducibility of antioxidant systems with exercise

It is important to understand alterations and adaptations of skeletal muscle antioxidant systems resulting from both acute and chronic exercise. Changes that occur under these conditions reveal the physiological strategies in skeletal muscle for dealing with oxidant stress. It is important to keep in mind that changes in each enzyme or substrate contributes to skeletal muscle antioxidant system status.

Acute exercise significantly decreases the GSH content and increases GSSG in working muscle (45). In addition, Ji et al. (68) have found a decrease in deep vastus lateralis total protein sulfhydryl content after treadmill run to exhaustion in rats. Vitamin E content is reported to be unchanged (101) with acute exercise. Glutathione peroxidase activity levels have been reported by Salminen and Vihko (101) to increase above control levels in mouse red vastus lateralis following exhaustive treadmill exercise. Ji et al. (68) have reported similar findings in the untrained rats for the soleus but not for the vastus lateralis or in trained rats. Enhancement of superoxide dismutase has not been reported with acute exercise, but Laughlin et al. (76) found an increase in SOD in untrained red gastrocnemius following ischemia reperfusion. Skeletal muscle catalase activity has been described by Alessio and Goldfarb (2) as increasing with 20 minutes of treadmill running in trained rat, but not untrained, red and white vastus lateralis. Thus, a reduction in reduced glutathione content and possible "upregulation" of antioxidant enzymes occurs in working skeletal muscle as a result of a bout of acute exercise. Differences in reported responses of the antioxidant enzymes may be a result of state of training, type of muscle examined, exercise protocol, and the particular enzyme in question.

Skeletal muscle glutathione peroxidase has been reported to increase (67,76) following endurance training. Mn-dependent superoxide dismutase has been described by Higuchi et al. (56) as inducible in the soleus and the red and white gastrocnemius following 3 months of endurance training; Cu/Zn-SOD demonstrated an increase in the soleus but not the red or white gastrocnemius. Quintanilha (95) found elevated cytosolic levels of SOD in the rat vastus lateralis following training. Girten et al. (43) and Jenkins (65) demonstrated an increase in total SOD in the rat soleus after 11 and 12 weeks respectively of endurance training. In contrast, Alessio and Goldfarb (2) and Ji et al. (67) found no alteration in SOD activity with endurance training in red, white vastus and whole hindlimb, respectively in the rat. Catalase has been reported to increase (43), remain constant (3,56) or decrease (76) in skeletal muscle following endurance training. Overall, a positive correlation between the percentage of oxidative fibers in a locomotor skeletal muscle group and antioxidant enzyme levels (SO>FOG>FG) has recently been described by Laughlin et al. (76). Data from our laboratory reveal a similar pattern (unpublished observations). Salminen and Vihko (101) and Gohil et al. (44) report unaltered quadriceps Vitamin E status following treadmill endurance training in the mouse, whereas Quintanilha (95) found a reduction in Vitamin E in the vastus lateralis after 10 weeks of training in the rat.

An important antioxidant defense response with endurance training may be the increase in mitochondrial volume (25), i.e., an increase in the number of mitochondia/muscle cell volume. The result is a reduced flux and lesser ubiquinone reduced state in trained muscle mitochondria at rest and during exercise. It is then probable that "metabolic leaks" and therefore free radical release are reduced. In fact, mitochondrial volume and markers such as citrate synthase and ubiquinones increase with endurance training at a faster rate than the antioxidant enzymes (44). Yet lipid peroxidation is decreased following endurance training. Further, induced selenium deficiency in rats which markedly lowers glutathione peroxidase activity results in a compensatory increase in mitochondria volume (72). Collectively, these observations support the notion that free radical signals play a role in active skeletal muscle mitochondrial biogenesis with endurance training.

Free Radical Toxicity and Skeletal Muscle Damage

Oxyradical toxicity occurs when the production of free radicals overwhelms the capabilities of the anitoxidant systems in an organ system. Such a scenario may occur in skeletal muscle with ischemia/reperfusion injury, inflammation, delayed-onset muscle soreness, muscular dystrophy, as well as exercise. Interest in free radical toxicity by exercise physiologists stems from evidence which indicates that lipid peroxidation is increased, skeletal muscle structure disrupted, activity of certain metabolic enzymes reduced, and sarcoplasmic reticulum and mitochondrial function impaired with acute, strenuous exercise. It is important to emphasize that potential contributory mechanisms in inducing skeletal muscle pathology with acute exercise are interactive. Moreover, contribution of mechanistic factors depends on fiber type composition, mode of exercise, intensity and duration of exercise, training state, muscle recruitment pattern, etc. A review of the literature indicates that a) mechanical disruption, b) Ca^{2+} homeostasis, c) arachidonic acid metabolites (leukotrienes and prostaglandins), as well as d) free radical production and antioxidant status act in an integrated manner in mediating exercise-induced muscle pathology (5,14,34,68). Exercise-induced skeletal muscle pathology may result in one or more of the following: membrane disruption and peroxidation, decrease in the ability of the sarcoplasmic reticulum to sequester Ca^{2+}, disturbances in Ca^{2+} homeostasis, mitochondrial swelling, mitochondrial disruption and loss, inactivation of metabolic enzymes, release of metabolic enzymes into the circulation, and myofibril disruption and destruction.

Eccentric Exercise

It now appears that damage involving lengthening contractions (eccentric exercise) such as in downhill running involves different distinct mechanisms and phases. Initial myofibril damage appears to be mediated primarily by disturbances in Ca^{2+} homeostasis primarily, but also by free radical production (5,34,128). Intracellular enzyme efflux has been demonstrated by Duncan and Jackson to be dependent primarily on phospholipase A_2 activity and leukotriene production (34). Subsequent proteolytic activity (24-48 hours post-exercise) is mediated by free radicals, leukocyte infiltration, and Ca^{2+}-activated proteases (5,128). The proteolytic phase may be thought of as the first step in muscle injury repair, as damaged skeletal muscle sarcomeres are digested.

Biochemical Alterations with Acute Exercise

A number of reports have cited biochemical alterations, which could be critical in skeletal muscle contractile function, as a result of acute exercise. Inactivation of metabolic enzymes, damage to cellular membrane lipids, mitochondrial dysfunction, and sarcoplasmic reticulum dysfunction have been reported to occur in locomotor muscle with an acute bout of exercise (3,8,13,14,31,64,68). It seems to be a reasonable assumption that if free radicals are contributory to fatigue and other skeletal muscle pathology, those affects would likely be manifested as physiologically detrimental alterations such as cellular membrane damage and metabolic machinery downregulation. Such "metabolic" damage may limit the production and utilization of ATP, thus limiting work during exercise.

Ji et al. (68) have observed significant reductions in citrate synthase, cytochrome oxidase, and malate dehydrogenase activity in untrained rats following 60 minutes of treadmill running at 27 $m \cdot min^{-1}$ and 15% grade; reduction in citrate synthase and malate dehydrogenase levels were less in trained rats at this work load. Boström et al. (8) have reported marked reductions in hexokinase and phosphofructokinase levels in rats after exhaustive swimming while a trend towards a decrease in cytochrome oxidase activity was not significant. Dohm et al. (31) have noted significant reductions in NAD^+-specific isocitrate dehydrogenase, succinate dehydrogenase, and cytochrome oxidase following exhaustive treadmill running in trained rats, but not untrained rats; however the untrained rats were exercised at a much lower treadmill speed (18 $m \cdot min^{-1}$ vs. 43 $m \cdot min^{-1}$) and were not acclimated to treadmill running.

Increases in stable lipid peroxidation products measured as cellular malondialdehyde following acute exercise have been reported to increase by Brady et al. (13) and Jenkins et al. (65), but not Ji et al. (68). Alessio and Goldfarb (2) have found an increase in malondialdehyde content of white, but not red, skeletal muscle with acute exercise in both trained and untrained rats. It should be noted here that data reported for malondialdehyde by Ji et al. (68) were for red vastus lateralis only. Also, the mechanisms for both lipid peroxidation and enzymatic downregulation are parallel and interactive but not obligatory. In addition, Byrd et al. (14) have noted an acute exercise duration dependent diminishment in rate of Ca^{2+} uptake in crude homogenates and purified sarcoplasmic reticulum vesicles indicating SR dysfunction. Thus, free radical production during acute exercise can result in enzymatic and cellular membrane alterations in structure and function. It is likely that intensity of exercise, duration of exercise, state of training, recruitment of the muscle in question during exercise, and antioxidant status all contribute to the degree of metabolic damage sustained by a muscle with a bout of acute exercise.

It is not surprising that metabolic enzymes are not exempt from oxidative damage due to free radicals. Inactivation of mitochondrial enzyme activity may be due to mitochondrial membrane disruption, depression of protein turnover, and direct interaction of free radicals on enzymatic protein structure. However, Ji et al. (68) noted no mitochondrial leaking, as measure by the presence of citric acid cyle enzyme in the cytosolic fraction, in rat locomotor muscle following exhaustive treadmill exercise. Also, Terjung et al. (120) found no change in mitochondrial yield following two hours of running trained rats to exhaustion. The authors attributed exhaustion in this instance to glycogen depletion. Disturbances in cellular homeostasis and mechanical perturbation of the muscle may activate protease and lysosomal activity (68). Protein tryptophan, tyrosine, histidine, methionine, and cysteine residues are susceptible to free radical attack. The cysteine sulfhydryl group (-SH; thiol) is especially vulnerable to peroxide oxidation (49,68). Intact sulfhydryl groups are essential for the catalytic properties of many metabolic enzymes (68). Exposed enzyme sulfhydryl groups may be oxidized to form disulfide bonds or sulfonic acid. Ji et al. (68) have found that a portion of the enzymatic downregulation or inactivation with acute exercise is due to oxidation of sulfhydryl groups. In addition, it is likely that cellular proteins which are substantially altered, as by free radical damage, are more susceptible to proteolytic and lysosomal degradation (97). Lipid peroxidation can produce stable products which can be measured systemically in expired air (pentane and ethane) or locally in tissue samples (malondialdehyde) (64).

Aging and Respiratory Muscle Considerations

Currently there is a paucity of literature concerning antioxidant status and exercise induced free radical damage in skeletal muscle with aging. Further information is lacking in terms of free radical production and management in respiratory muscles. Ji et al. (66) have reported that in sedentary aged Wistar rats that skeletal muscle lipid peroxidation and antioxidant enzyme levels are increased. However, this is not consistent with data collected concerning age-related alteration in lipid peroxidation and glutathione peroxidase levels by Starnes et al. (117) and Zhang et al. (129) respectively. Wu et al. (126) found lower GPX activity in the vastus lateralis of untrained 27 vs. 5 month old Fischer-344 rats. However, the vastus lateralis GPX levels of 27 month old rats after 10 weeks of training was equivalent with the 5 month untrained group (126). Zerba and Faulkner (128) have found an increased contribution of free radical mechanisms to muscle damage following lengthening contractions in older animals. However, it is not known if older animals are more susceptible to muscle myofibril and metabolic damage due to an acute bout of uphill running.

Moreover, there are no reports concerning respiratory muscles in senescent animals concerning acute exercise induced alterations in lipid peroxidation and metabolic enzyme status. Although free radical related cytotoxicity *in vitro* (105) and fatigue, under hyperoxic conditions (107), have been reported in the diaphragm, specific biochemical alterations such as lipid peroxidation and metabolic enzyme alterations following exercise have not been investigated. Moreover, such responses in senescent animals are unknown. Work of breathing increases with age in humans (21,35) as a result of decreased mechanical compliance and increase airway resistance. We have observed in our laboratory (unpublished data) that the diaphragm possesses higher antioxidant enzyme levels when compared with the plantaris. However, the significance of this protection is, as yet, unclear including any possible interaction with age.

METHODS

Animals and Exercise Protocol

To study the effects of an acute bout of submaximal exercise on free radical mediated alterations in metabolic and antioxidant enzymes as well as lipid peroxidation in the diaphragm, young and old rats performed 40 minutes of treadmill exercise using the following procedures. Twenty 24 month old and twenty 4 month old female Fisher 344 rats who demonstrated willingness to run on a treadmill comprised the aged and young groups, respectively. Inbred animals were purchased from Harlan Sprague-Dawley (NIA colony), and were housed and cared for in accordance with American Physiological Society policy. All animals participated in a treadmill habituation period which consisted of 10 consecutive days of treadmill running at 10% grade for 5 min•day^{-1}. During the acclimation period, older animals ran at a treadmill speed of 10-15 m•min^{-1}; the younger animals were acclimated at a treadmill speed of 16-22.5 m•min^{-1}. Data from Ji et al. (68) as well as our laboratory (unpublished observations) reveal that skeletal muscle metabolic enzyme are unaffected by such a treadmill habituation protocol. Following the acclimation period, 20 animals in each age group were randomly assigned to either exercise, old exercised (OE: n=10) and young exercised (YE: n=10); or control (old control (OC: n=10) and young control: (YC: n=10)) groups.

The acute bout of exercise was performed on each animal 48 hours after the final day of acclimation. The treadmill speed and grade were designed to require ~75% of the age-group $\dot{V}O_2max$. These workrates were chosen based upon treadmill maximal oxygen consumption and O_2 cost of running data recently collected in our laboratory in untrained young adult and aged female Fischer-344 rats from the same colony and inbred strain. The acute exercise protocol consisted of 40 minutes of uphill treadmill running at ~14.5

m•min^{-1} (10% grade) for the 24 month old rats and ~22.0 m•min^{-1} (10% grade) for the young group. Mild electric shock (<1 mA) was used to encourage animals to run. Four, 24 month and two, 4 month old animals could not complete the full 40 minutes of treadmill exercise. However, all animals completed over 30 minutes of work and were included in data analysis.

Immediately following completion of treadmill exercise, each animal was euthanized with sodium pentobarbital (100 mg•kg^{-1} i.p.). At the time of sacrifice, control animals were euthanized as matched pairs with the exercised animals. The costal and crural diaphragm, plantaris, gastrocnemius and soleus muscles were quickly removed. These tissues were immediately placed in ice-cold Ringer solution and dissected from fat and connective tissue. At that time the red and white portions of the gastrocnemius were separated. All tissues were then frozen immediately in liquid nitrogen and stored at -80°C until analyses. Time for muscle excision, dissection, and freezing was <5 minutes.

Biochemical Preparation and Assays

Homogenization Procedure

Tissues were homogenized in 100 mM potassium phosphate buffer (pH=7.4) containing 0.05% bovine serum albumin (BSA), 10 mM EDTA, 0.13 mM butylated hydroxytoluene (BHT), and 0.13 mM desferrioxamine. The addition of EDTA, BHT (antioxidant), desferrioxamine (iron chelator) to the buffer was designed to minimize oxidation of lipid membranes, enzymes and substrates during the homogenization and assay procedures (51). Pilot data revealed higher enzyme activity levels for the above buffer solution vs. potassium phosphate buffer and BSA alone. Homogenization was performed at 0-5°C and included 15 second treatment with a Ultra-Turrax T25 tissue homogenizer (IKA-Works, Cincinnati) followed by 10 passes with a tight fitting glass-on-teflon Potter-Elvehjem homogenizer. The homogenates were the centrifuged at 3°C at 400g for 10 minutes to remove cellular debris. This procedure has been demonstrated as proficient in mitochondrial disruption of rat skeletal muscle (77).

Biochemical Assays

Phosphofructokinase (PFK) activity was determined using the procedure by Shonk et al. (108). Mitochondrial enzymes activities were performed as follows: citrate synthase (CS) activity was assayed using the method of Srere (116); 3-hydroxyacyl-CoA-dehydrogenase (HADH) activity was determined via the technique of Bass et al. (7); and succinate dehydrogenase (SDH) activity was assayed using the method of Singer (109). The activity of antioxidant enzymes glutathione peroxidase and superoxide dismutase were determined using the techniques of Flohé and Günzler (36) and Marklund and Marklund (81), respectively. Lipid peroxidation was estimated via thiobarbituric acid assay for malondialdehyde (MDA) as described by Ohkawa et al. (88). Protein content was assessed via dye-binding using the Bradford technique (12). If enzyme inactivation occurred dithiothreitol (a thiol reagent) was administered in select samples to assess potentially reversible inhibition. Selected muscle samples were incubated for 25 minutes in 1mM dithiothreitol and reassayed. Diothiothreitol can reduce reversible disulfide cross-linking to reform sulfhydryl groups in enzymes (68). Disulfide cross-linking may occur via thiol/disulfide exchange or oxidation mechanisms, and can alter protein 3-dimensional conformation as well as enzymatic active sites.

Data Analysis

A three-way (6x2x2) analysis of variance (ANOVA) for tissues, age, and exercise treatment was performed on the obtained data to determine if significant main and interactive effects exist, with Fisher-PLSD test used post-hoc where appropriate. In addition, Pearson-product moment correlation coefficient analyses were performed among all measured variables across all tissues and experimental groups. Level of significance was set at <0.05. Data were reported in figures as the means ± standard error of the means.

RESULTS

Body weights (mean ± SEM) of the control animals did not differ from the exercised animals in either the 4 month (YC = 207.6 ± 11.8g; YE = 200.3 ± 17.0g or the 24 month (OC = 296.3 ± 23.4g; OE = 287.2 ± 20.7g) old rats. However the 24 month old rats had significantly ($P<0.001$) higher body weights than the 4 month old rats when data were pooled by age.

Bioenergetic Enzymes

Phosphofructokinase activity displayed significant main effects for exercise, age, and muscle. In the white gastrocnemius the exercised groups of both 4 month and 24 month had significantly lower PFK values than controls (Figure 1). PFK activities in the white gastrocnemius were significantly reduced 32.6% and 60.2% by exercise in the young and old animals, respectively. The older exercised group (OE) had significantly lower activities (by 41.2%) in the red gastrocnemius compared to controls (OC) as well. The YE group displayed a trend (20.8% lower) toward decreased PFK activity levels in the red gastrocnemius than control (YC) but was not significant. Note that a survey of individual responses revealed that some YE red gastrocnemius muscles displayed "downregulation" (i.e., ≥ 2 std. dev. from YC mean) whereas others did not. A portion (4 in red gastroc.; 4 in white gastroc) of the muscle samples which displayed PFK inactivation were incubated in 1mM dithiothreitol and reassayed. Dithiothreitol had minimal impact upon PFK activities in the exercised gastrocnemius samples reassayed; only ~10% recovery was noted. No significant exercise effects on PFK activity existed in the costal or crural diaphragm, the plantaris, or the soleus. PFK activity levels were higher for the costal and crural diaphragm than in the soleus; however PFK activities in the costal and crural diaphragm were significantly lower than in the red or white gastrocnemius.

In contrast, measurements of citrate synthase and 3-hydroxyacyl-CoA-dehydrogenase enzyme activities (Figures 2 and 3) displayed no significant effects due to acute treadmill exercise in costal diaphragm, crural diaphragm, plantaris, red gastrocnemius, white gastrocnemius or soleus. Citrate synthase activity was significantly lower for the red gastrocnemius in the 24 month when compared to the 4 month old rats. No main effect for age was found for 3-hydroxyacyl-CoA-dehydrogenase. In addition, no alterations in SDH activity with acute exercise were noted in the costal or crural diaphragm (data not shown).

Antioxidant Enzymes

Data for glutathione peroxidase activities are shown in Figure 4. A significant overall age and tissue effect were found for GPX. Twenty-four month old animals possessed significantly higher GPX activities than young animal in the plantaris and white gastrocnemius. The costal and crural diaphragm had GPX activity levels approximately 300% higher than the plantaris. In addition there was a large, significant discrepancy for GPX levels among the remaining locomotor muscles with soleus > red gastrocnemius > white gastrocnemius. Significantly higher GPX activities were found in the plantaris for both exercise groups (OE and YE) when compared to the respective controls (OC and YC). In addition, white gastrocnemius YE glutathione activity levels were significantly higher than YC. In contrast, GPX enzyme activities were not significantly different in the white gastrocnemius between OE and OC. A significant age x exercise interaction for GPX activity existed in the red gastrocnemius. No alterations due to exercise or age for GPX were found in the costal or crural diaphragm. Further, acute exercise had no effect on soleus GPX levels for both 4 and 24 month old animals.

ANOVA demonstrated significant exercise and tissue main effects for total superoxide dismutase activity (Figure 5); no significant main effects for age (4 mo. vs. 24 mo.) were noted. Overall exercised animals displayed significantly higher mean SOD activities than controls. However, subsequent post-hoc analysis with respect to SOD activity revealed no significant differences between corresponding exercised and control groups within any specific tissue; however, the largest exercise-induced trends in SOD activity were in the following YE groups when compared to YC: the crural diaphragm (26.6% increase), plantaris (12.6% increase), white gastrocnemius (12.5% increase), and soleus (13.9% increase).

In addition, citrate synthase/glutathione peroxidase ratio was significantly lower in the old rats vs. young rats for the costal diaphragm, plantaris, white gastrocnemius, red gastrocnemius, and soleus. Thus, age appears to have an antioxidant enzyme-specific effect in the skeletal muscles measured.

Lipid Peroxidation

Malondialdehyde levels in the tissues studied are presented in Figure 6. Significant overall exercise and tissue main effects were revealed for MDA. In addition, a significant overall age x tissue interaction was present. In the costal diaphragm YE had greater MDA levels than YC whereas no effect was found for the old rats. Also, red gastrocnemius MDA levels were higher for YE when compared to YC; again, no exercise effect was found between OE and OC. The trend towards higher MDA levels in YE vs. YC was not significant. Note that tissue means for MDA displayed patterns similar to that found for GPX in locomotor muscles. Finally, MDA levels for the diaphragm were significantly less than in the red gastrocnemius.

Correlations

A correlation coefficient matrix for all measure variables is found in table 1 for combined data across all tissues assayed (costal and crural diaphragm, plantaris, white and red gastrocnemius, and soleus). All correlation coefficients discussed below were significant ($P<0.05$). The high degree of relationship noted among the mitochondrial citric acid cycle (CS, SDH) and ß-oxidation (HADH) enzyme activities (CS vs. SDH: $r=.882$; CS vs. HADH: $r=.916$; SDH vs. HADH: $r=.895$) is similar to numerous previous reports involving skeletal muscle. The activity of the rate limiting enzyme for glycolysis, PFK, was negatively correlated with all other markers. The highest degree of association for PFK vs. other markers was found in comparison with the antioxidant markers GPX ($r=-.607$) and SOD ($r=-.522$). A comparison of GPX and SOD revealed a significant correlation coefficient across all tissues of $r = 0.743$. The lipid peroxidation marker MDA possessed significant correlations with GPX ($r=.674$), SOD ($r=.373$), and PFK ($r=-.394$), but not HADH, CS, or SDH. As glutathione peroxidase can reduce hydroperoxides produced with lipid peroxidation, it is not surprising that GPX and MDA have a moderately high degree of relationship ($r=.678$). In addition, significant correlations across all tissues were found for SOD when compared to the mitochondrial bioenergetic enzymes CS ($r=.619$), SDH ($r=.543$), and HADH ($r=.743$). Finally, significant correlations across all tissues were found for GPX when compared to the oxidative metabolic enzymes CS ($r=.297$), SDH ($r=.299$), and HADH ($r=.561$). Thus, in general, the activity of SOD is more representative of markers of oxidative metabolism as well as GPX activity; and GPX activity is more closely associated with the measured marker of lipid peroxidation as well SOD activity.

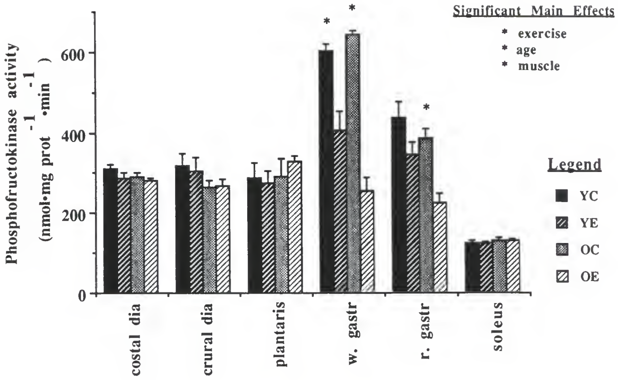

Figure 1. Phosphofructokinase activities (mean ± SEM) expressed in nM substrate converted per minute per mg protein for costal (costal dia) and crural (crural dia) diaphragm, plantaris, white gastrocnemius (w. gastr), red gastrocnemius (r.gastr), and soleus muscles for young control (YC), young exercised (YE), old control (OC), and old exercised (OE). * denotes significantly different from exercised (P<0.05).

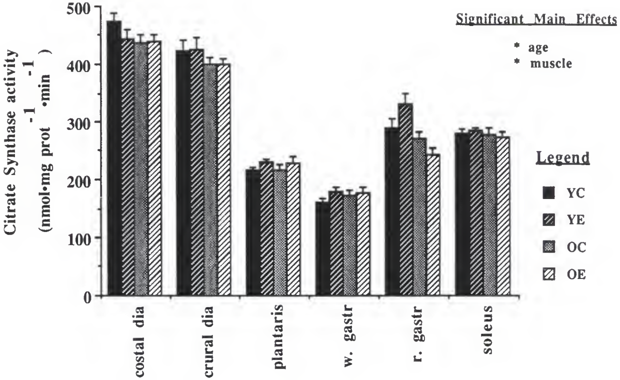

Figure 2. Citrate synthase activities (mean ± SEM) expressed in nM substrate converted per minute per mg protein for costal (costal dia) and crural (crural dia) diaphragm, plantaris, white gastrocnemius (w. gastr), red gastrocnemius (r.gastr), and soleus muscles for young control (YC), young exercised (YE), old control (OC), and old exercised (OE).

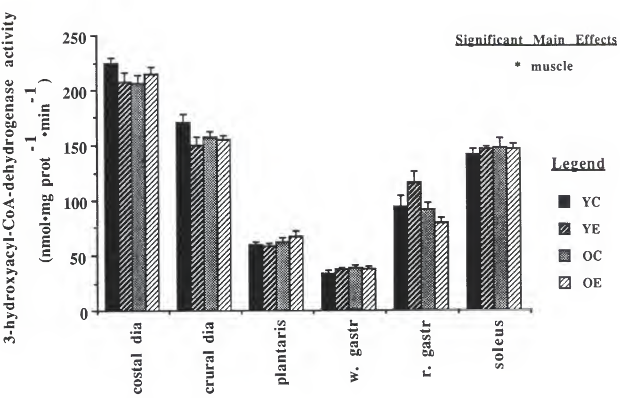

Figure 3. 3-Hydroxyacyl-CoA dehydrogenase activities (mean ± SEM) expressed in nM substrate converted per minute per mg protein for costal (costal dia) and crural (crural dia) diaphragm, plantaris, white gastrocnemius (w. gastr), red gastrocnemius (r.gastr), and soleus muscles for young control (YC), young exercised (YE), old control (OC), and old exercised (OE).

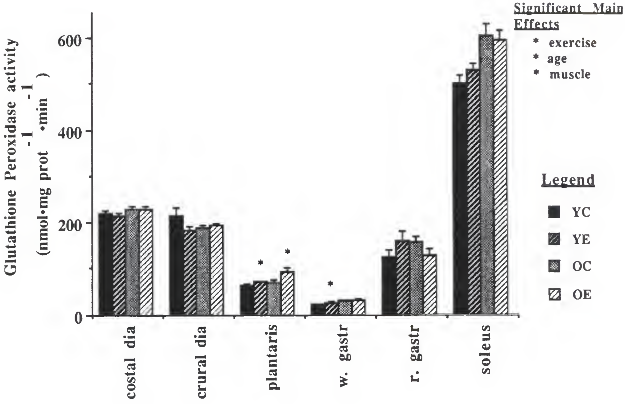

Figure 4. Glutathione peroxidase activities (mean ± SEM) expressed in nM substrate converted per minute per mg protein for costal (costal dia) and crural (crural dia) diaphragm, plantaris, white gastrocnemius (w. gastr), red gastrocnemius (r.gastr), and soleus muscles for young control (YC), young exercised (YE), old control (OC), and old exercised (OE). * denotes significantly different from exercised ($P<0.05$).

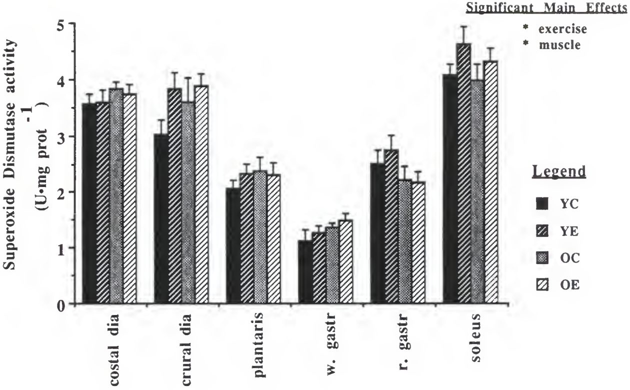

Figure 5. Superoxide dismutase activities (mean ± SEM) expressed in nM substrate converted per minute per mg protein for costal (costal dia) and crural (crural dia) diaphragm, plantaris, white gastrocnemius (w. gastr), red gastrocnemius (r.gastr), and soleus muscles for young control (YC), young exercised (YE), old control (OC), and old exercised (OE).

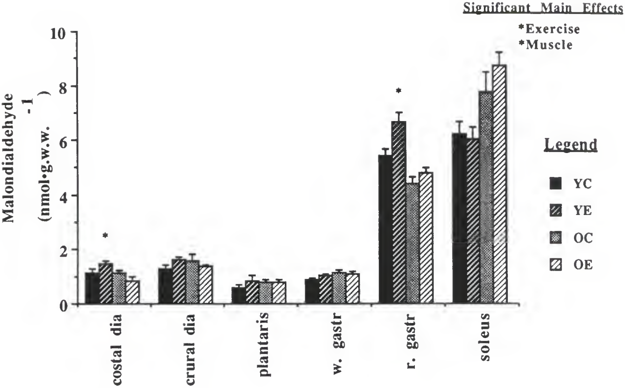

Figure 6. Malondialdehyde concentrations (mean ± SEM) expressed in nM per gram wet weight for costal (costal dia) and crural (crural dia) diaphragm, plantaris, white gastrocnemius (w. gastr), red gastrocnemius (r.gastr), and soleus muscles for young control (YC), young exercised (YE), old control (OC), and old exercised (OE). * denotes significantly different from exercised (P<0.05).

Table 1. Correlation coefficient matrix for malondialdehyde (MDA), glutathione peroxidase (GPX), superoxide dismutase (SOD), 3-hydroxyacyl-CoA-dehydrogenase dehydrogenase (HADH), citrate synthase (CS), succinate dehydrogenase (SDH), and phosphofructokinase (PFK). Data is combined for costal and crural diaphragm, red and white gastrocnemius, plantaris and soleus. All correlations are statistically significant except for MDA vs. HADH, MDA vs. SDH, and MDA vs. SOD.

Correlation matrix

	MDA	GPX	SOD	HADH	CS	SDH	PFK
MDA	1						
GPX	.678	1					
SOD	.377	.743	1				
HADH	.132	.561	.743	1			
CS	-.045	.297	.619	.916	1		
SDH	-.088	.299	.543	.895	.882	1	
PFK	-.392	-.607	-.522	-.362	-.225	-.16	1

DISCUSSION

The current data demonstrate that metabolic enzyme activities (PFK, CS, HADH, SDH) of both the costal and crural diaphragm are protected from downregulation in both young and old rats during 40 minutes of treadmill running (10% uphill grade) at ~75% VO_2max. In contrast, significant downregulation of phosphofructokinase activity (not reversible by dithiothreitol) occurred with this level of acute exercise in the red (OE) and white (YE and OE) gastrocnemius but not the plantaris or soleus. The degree of downregulation of PFK was greater in the old exercised than in young exercised groups when compared to age-matched control animals. The mitochondrial metabolic enzyme activities measured (CS and HADH) were not compromised by acute exercise for both age groups in costal and crural diaphragm, plantaris, white and red gastrocnemius, and soleus. Finally, tissue malondialdehyde is increased with acute treadmill exercise to a lesser extent in old than in young rats. A brief discussion of the preceding principal findings follows.

Mean levels of the lipid peroxidation marker, malondialdehyde, were increased in the YE costal diaphragm by 31.4% when compared with YC (Figure 6); however, no such exercise-induced increases in diaphragmatic MDA levels were found in OE. This suggests that in young animals an exercise-induced increase in lipid peroxidation in the diaphragm (a likely a result of an elevation in free radical production) occurs with 40 minutes of exercise at ~75% VO_2max. Concomitantly small downward trends (~7%) in YE when compared to VC for PFK, CS, and HADH with the current acute exercise protocol for the diaphragm. Although the aforementioned trends were neither statistically or physiologically significant, it is unknown if downregulation of metabolic enzymes is manifested in the diaphragm at higher work intensities and/or duration. Further study is warranted.

Overall, the data indicate an effective antioxidant strategy in the rat diaphragm to quench free radical production and to protect metabolic enzymes from damage with the current exercise regimen. Bioenergetic enzyme protection from exercise-induced alterations, noted in the current investigation, in the diaphragm stems from at least three factors: 1) high antioxidant enzyme status (Figures 4 and 5); 2) relatively large mitochondrial volume in the diaphragm (as indicated by high CS and HADH activities: Figures 2 and 3) compared to locomotor muscle; and 3) oxygen cost of breathing in the diaphragm during the prescribed exercise vs. oxygen cost of running in the locomotor muscles. A large mitochondrial volume (i.e., high number of mitochondria) in muscle provides indirect protection as suggested by Davies et al. (25). A higher mitochondrial volume per cross-sectional area of muscle results in a lower O_2 flux per individual mitochondria at any given tissue oxygen consumption. As free radical production by the electron transport chain is directly related to mitochondrial O_2 flux and concomitant ubiquinone reduction state (10), a given tissue oxygen consumption in muscles with higher mitochondrial volume theoretically translates into a lower release of free radicals.

Although it appears that major energy producing pathway enzymes were not compromised in the diaphragm, sarcoplasmic reticulum function, membrane transport proteins, Ca^{2+} homeostasis, and whole mitochondrial function were not assessed in the diaphragm. Thus, it is not possible to state that the metabolic system of the diaphragm *in toto* was not "injured" with the prescribed bout of acute exercise.

Partial inactivation of phosphofructokinase with acute exercise (Figure 1) in the white and red gastrocnemius, but not the soleus (as well as the diaphragm) indicates tissue specificity of "metabolic injury" at the exercise intensity and duration used in the current study. The tissue specific effects of PFK downregulation with acute exercise among the locomotor muscles measured are likely related to 1) tissue specific activities of the antioxidant enzymes superoxide dismutase (Figure 5) and glutathione peroxidase (Figure 4), and 2) degree of muscle recruitment and consequent metabolic load during the prescribed level and intensity of exercise. The high levels of SOD and GPX found in the soleus, which has marked increases in blood flow during prolonged work (74), may provide substantial protection of metabolic enzymes from free radical toxicity. The red gastrocnemius is heavily recruited during submaximal work (74,75), but has lower SOD and GPX activities (Figures 5 and 4).

In addition, elevated malondialdehyde levels in the red gastrocnemius were noted in YE when compared to YC (Figure 6). Thus, sustained submaximal exercise greatly stresses the oxygen intermediate management system of the red gastrocnemius. Blood flow and recruitment increases markedly in the white gastrocnemius (74) during heavy work, but less so with moderate sustained work. However, antioxidant enzyme activities (SOD and GPX) as well as mitochondrial enzyme activities (CS and HADH) were found to be very low in the white gastrocnemius. The combination of low antioxidant enzyme activity levels coupled with a low mitochondrial volume could make the white gastrocnemius highly susceptible to free radical toxicity with strenuous exercise. Further, importance of antioxidant enzyme levels on is illustrated by the current data set, as follows. The degree of PFK downregulation (Figure 1) was inversely related with the antioxidant status of the white gastrocnemius, red gastrocnemius and soleus. Thus, the susceptibility of PFK downregulation in locomotor muscle, with the current exercise protocol, as a function of fiber type could be expressed as the following: FG > FOG > SO. Allosteric modifiers of PFK *in vivo* (H^+, ATP, ADP, citrate, cellular oxidized thiol/disulfide ratio) may further alter PFK downregulation with acute exercise.

Potentially, phosphofructokinase is highly susceptible to free radical attack and subsequent proteolytic degradation as it has a high number of exposed sulfhydryl groups (7-16) in mammalian species (73). Dithiothreitol did not appear to reactivate down regulated PFK activity in the gastrocnemius; this could indicate that the phenomenon is not exclusively a result of reversible thiol/disulfide exchange, an important metabolic control mechanism for PFK. Therefore, exercise may have elicited a specific protein loss in the gastrocnemius via Ca^{2+}-activated proteolytic degradation (23) of damaged PFK molecules and/or irreparable damage (via free radicals mediated biochemistry and/or another mechanism) specific to PFK. Recent evidence by Byrd et al. (14) indicates a diminished ability of gastrocnemius and vastus lateralis sarcoplasmic reticulum to sequester Ca^{2+} during sustained (>20 minutes) work in rats. Resultant alterations in cellular Ca^{2+} homeostasis could theoretically exacerbate and/or initiate free radical pathology in skeletal muscle as well as activate Ca^{2+}-dependent proteases (16,23) which could irreversibly degrade free-radical and/or age weakened proteins.

As previously mentioned, reduction of PFK activity in the gastrocnemius (Figure 1) with acute treadmill exercise was greater for 24 month old rats when compared with 4 month old rats. Although both YE and OE exercised at ~75% of respective age group VO_2max, the absolute treadmill speeds (thus workrates and metabolic cost) were higher for the YE group (14.5 $m \bullet min^{-1}$) when compared to OE (22.0 $m \bullet min^{-1}$). Indeed, exercise-induced alterations in antioxidant enzyme activities (SOD and GPX) and lipid peroxidation (MDA) were more pronounced in gastrocnemius YE than OE when compared to respective control groups. This evidence suggests that free radical production may be more closely related to absolute than to relative workrates.

It is tempting to speculate that the heightened susceptibility of gastrocnemius PFK to exercise-induced downregulation in senescent animals may be related to a "structurally weak" native enzyme. Senescent animals produce markedly more proteins which have errors in amino acid sequencing (98). Therefore, it is possible that in older animals possess a greater number of PFK molecules with error(s) in native protein sequence and structure which have some or all of the activity. Further, such "weakened" proteins could be more susceptible to free radical attack and protein degradation. Additional research is needed to unravel this puzzle.

Although, PFK activity was inhibited in the gastrocnemius, the mitochondrial (CS and HADH) enzyme activities were not affected by a bout of acute exercise. This indicates an bioenergetic enzyme-specific downregulation was induced by the prescribed treadmill exercise in young and old female Fischer-344 rats. Other authors have reported inhibition of mitochondrial enzymes (8,31,46,68) and mitochondrial function (32,46) with a bout of acute exercise. Discrepancies between the current study and other investigations which have reported inactivation of mitochondrial enzymes may be explained as follows. Those studies that have found disturbances in mitochondrial function and mitochondrial enzyme activity employed protocols which elicit exhaustion in rats (68) or exercise protocols of higher intensity (46) and duration (8,31,68). Further, state of training influences susceptibility of mitochondrial enzymes to downregulation (8,68). Finally, metabolic enzyme-specificity of downregulation with acute exercise may be related to enzyme location: PFK is a cytosolic enzyme; CS, SDH, and HADH are mitochondrial. In such a model, free radical production during acute exercise in active muscle by the sarcoplasmic reticulum and/or muscle microvascular endothelium (via xanthine oxidase: ref. 110) would have significant functional importance in damaging cytosolic components.

In summary, phosphofructokinase activity was found to be inhibited with a bout of acute exercise in the white and red gastrocnemius in senescent female Fischer-344 rats. The degree of exercise-induced PFK downregulation in both white and red gastrocnemius was less in the young adult group. The degree of inhibition was greater in the white gastrocnemius when compared to the red gastrocnemius. It should be noted that *in vivo*, during exercise, PFK inhibition may be proportionally greater in YE and OE due to allosteric inhibition of both increased muscle [H^+] and more oxidized thiol/disulfide ratio. In addition, the diaphragm was adequately protected form metabolic enzyme downregulation at the current exercise intensity and duration. Finally, mitochondrial enzyme activities were not adversely affected with the prescribed bout of acute exercise in skeletal muscle.

SUMMARY AND CONCLUSIONS

Data from the current study revealed that the metabolic enzymes of both the costal and crural diaphragm are well-protected from inhibition in activity in both young and old rats as a result of 40 minutes of uphill treadmill running at ~75% of age-group $\dot{V}O_2max$. We speculate that the metabolic protection in the diaphragm is due to the relatively high antioxidant enzyme content, high mitochondrial content, and recruitment patterns during exercise. There is a sizable inactivation with acute exercise of phosphofructokinase in the red and white gastrocnemius. The level of inactivation is greater in the old exercised than in the young exercised. Dithiothreitol has little effect on exercise-induced PFK inactivation. This indicates that PFK inactivation for this level of exercise in the gastrocnemius is primarily not related to thiol/disulfide exchange. Mitochondrial enzyme activities measured were not compromised by acute exercise for both age groups in costal and crural diaphragm, plantaris, white and red gastrocnemius, and soleus. We propose that this is related to intensity and duration of the exercise protocol used.

REFERENCES

1. Alessio, H. M. and R. G. Cutler. Evidence that DNA damage and repair cycle activity increases following a marathon race. Medicine and Science in Sports and Exercise. 22: (Abstr) S126, 1990.

2. Alessio, H. M. and A. H. Goldfarb. Lipid peroxidation and scavenger enzymes during exercise: adaptive responses to training. Journal of Applied Physiology. 64: 1333-1336, 1988.

3. Alessio, H. M., A. H. Goldfarb, R. G. Cutler. MDA content increases in fast- and slow-twitch skeletal muscle with intensity of exercise in a rat. American Journal of Physiology. 255: C874-C877, 1988.

4. Amezcua, J. L., G. J. Dusting, R. M. J. Palmer, and S. Moncada. Acetylcholine induces vasodilation in the rabbit isolated heart through the release of nitric oxide, the endogenous nitrovasodilator. British Journal of Pharmacology. 95: 830-834, 1988.

5. Armstrong, R. B. Initial events in exercise-induced muscle injury. Medicine and Science in Sports and Exercise. 22: 429-435, 1990.

6. Balke, P. O., M. T. Snider, and A. P. Bull. Evidence for lipid peroxidation during moderate exercise. Medicine and Science in Sports and Exercise. 16: 181, 1984.

7. Bass, A., D. Brdiczka, P. Eyer, S. Hofer, and D. Pette. Metabolic differences of distinct muscle types at the level of enzymatic organizations. European Journal of Biochemistry. 10: 198-206, 1969.

8. Boström, S., M. Fahlén, Å. Hjalmarson, and R. Johansson. Activities of rat muscle enzymes after acute exercise. Acta Physiologica Scandinavica. 90: 544-554, 1974.

9. Boveris, A., E. Cadenas, and A. O. M. Stoppani. Role of ubiquinone in the mitochondrial generation of hydrogen peroxide. Biochemical Journal. 156: 435-444, 1976.

10. Boveris, A. and B. Chance. The mitochondrial generation of hydrogen peroxide. General properties and effect of hyperbaric oxygen. Biochemical Journal. 134: 707-716, 1973.

11. Boxer, L. A. The role of antioxidants in modulating neutrophil functional responses. Advances in Experimental Medicine and Biology. 262: 19-33, 1990.

12. Bradford, M. A rapid sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding, Analytical Biochemistry. 72: 248-254, 1976.

13. Brady, P. S., L. J. Brady, and D. E. Ullrey. Selenium, Vitamin E and the response to swimming stress in the rat. Journal of Nutrition. 109: 1103-1109, 1979.

14. Byrd, S. K., A. K. Bode, and G. A. Klug. Effects of exercise of varying duration on sarcoplasmic reticulum function. Journal of Applied Physiology. 66: 1383-1389, 1989.

15. Cadenas, E. Biochemistry of oxygen toxicity. Annual Review of Physiology. 58: 79-110, 1989.

16. Cadenas, E., A. Boveris, C. I. Ragan, and A. O. M. Stoppani. Production of superoxide radicals and hydrogen peroxide by NADH-ubiquinone reductase and ubiquinol-cytochrome c reductase from beef-heart mitochondria. Archives of Biochemistry and Biophysics. 180: 248-257, 1977.

17. Chance, B., H. Sies, and A. Boveris. Hydroperoxide metabolism in mammalian organisms. Physiological Review. 59: 527-605, 1979.

18. Chia, L. S., J. E. Thompson, and M. A. Moscallero. Disorders in human myelin induced by superoxide radical: an *in vitro* investigation. Biochemical and Biophysical Research Communication. 117: 141-146, 1983.

19. Chow, C. K. Interrelationships of cellular antioxidant defense systems. In: Cellular Antioxidant Defense Systems. Ed. by C. K. Chow. Boca Raton, FL: CRC Press, 1988, vol. II, pp. 217-237.

20. Cochrane, C. G., I. U Schrausfstatter, P. Hyslop, and J. Jackson. In: Oxygen Radicals and Tissue Injury. Ed. by B. Halliwell. Bethesda, MD: FASEB, 1988, pp. 49-54.

21. Davidson, J. K., K. Wasserman, G. Lillington, and R. Schmidt. Effect of aging on respiratory mechanics and gas exchange in rabbits. Journal of Applied Physiology. 21: 837-842, 1966.

22. Davies, K. J. A. Intracellular proteolytic systems may function as secondary antioxidant defense. An hypothesis. Journal of Free Radical Biology and Medicine. 2: 155-173, 1986.

23. Davies, K. J. A. Proteolytic systems as secondary antioxidant defenses. In: Cellular Antioxidant Defense Systems. Ed. by C. K. Chow. Boca Raton, FL: CRC Press, 1988, vol. II, pp.25-67.

24. Davies, K. J. A., S. W. Lin, and R. E. Pacifici. Protein damage and degradation by oxygen radicals: IV. Degradation of denatured protein. Journal of Biological Chemistry. 262: 9914-9920, 1987.

25. Davies, K. J. A., L. Packer, and G. A. Brooks. Biochemical adaptation of mitochondria, muscle, and whole-animal respiration to endurance training. Archives of Biochemistry and Biophysics. 209: 539-554, 1981.

26. Davies, K. J. A., A. T. Quintanilha, G. A. Brooks, and L. Packer. Free radicals and tissue damage produced by exercise. Biochemica et Biophysica Acta. 107: 1198-1205, 1982.

27. Del Maestro, R. F. An approach to free radicals in medicine and biology. Acta Physiologica Scandinavica. 92: 153-168, 1980.

28. Della Corte, E. and F. Stirpe. The regulation of rat liver xanthine oxidase. Biochemical Journal. 126: 739-745, 1972.

29. Deneke, S. M. and B. L. Fanburg. Regulation of cellular glutathione. American Journal of Physiology. 257: L163-L173, 1989.

30. Dillard, C. J., R. E. Litov, W. M. Savin, E. E. Dumelin, and A. L. Tappel. Effects of exercise, Vitamin E, and ozone on pulmonary function and lipid peroxidation. Journal of Applied Physiology. 45: 927-932, 1978.

31. Dohm, G. L., R. L. Huston, E. W. Askew, and H. L. Fleshood. Effects of exercise, training, and diet on muscle citric acid cycle enzyme activity. Canadian Journal of Biochemistry. 51: 849-854, 1973.

32. Dohm, G. L., R. L. Huston, E. W. Askew, and P. C. Weiser. Effects of exercise on activity of heart and muscle mitochondria. American Journal of Physiology. 223: 783-787, 1972.

33. Dohm, G. L., G. J. Kasparek, E. B. Tapscott, and H. A. Barakat. Protein metabolism during endurance exercise. Federation Proceedings. 44: 348-352, 1985.

34. Duncan, C. J. and M. J. Jackson. Different mechanisms mediate structural changes and intracellular enzyme efflux following damage to skeletal muscle. Journal of Cell Science. 87: 183-188, 1987.

35. Estenne, M., J. Yernault, and A. De Troyer. Rib cage and diaphragm-abdomen compliance in humans: effects of age and posture. Journal of Applied Physiology. 59: 1842-1848, 1985.

36. Flohé, L. and W. A. Günzler. Assays of glutathione peroxidase. Methods in Enzymology. 105: 114-121, 1984.

37. Floyd, R. A. Role of oxygen free radicals in carcinogenesis and brain ischemia. The FASEB Journal. 4: 2587-2597, 1990.

38. Foote, C. S., F. C. Shook, and R. B. Abakerli. Characterization of singlet oxygen. Methods in Enzymology. 105: 36-47, 1984.

39. Franceschi, C., A. Cossarizza, D. Monti, V. Comaschi, G. Farruggia, G. Sartor, and L. Masotti. Cellular defence mechanisms, cell death and aging: an integrated view. In: Protein Metabolism in Aging. Ed. by H.L. Segal, M. Rothstein, and E. Bergamini. New York: Wiley-Liss, pp. 395-405, 1990.

40. Fridovich, I. Overview: Biological sources of $O_2^{\bullet -}$. Methods in Enzymology. 105: 59-61, 1984.

41. Gayeski, T. E. J., R. J. Connett, and C. R. Honig. Oxygen transport in rest-work transition illustrates new functions for myoglobin. American Journal of Physiology. 248: H914-H921, 1985.

42. Gibson, Q. H. The reduction of methemoglobin in red blood cells and studies on the cause of idiopathic methemoglobinaemia. Biochemical Journal. 42: 13-23, 1948.

43. Girten, B., C. Oloff, P. Plato, E. Eveland, A. J. Merola, and L. Kazarian. Skeletal muscle antioxidant enzyme levels in rats after simulated weightlessness, exercise, and dobutamine. The Physiologist. 32 (suppl.): S2, 1989.

44. Gohil, K., L. Rothfuss, J. Lang, and L. Packer. Effect of exercise training on tissue vitamin E and ubiquinone content. Journal of Applied Physiology. 63: 1638-1641, 1987.

45. Gohil, K., S. Viguie, W. C. Stanley, G. A. Brooks, and L. Packer. Blood glutathione oxidation during human exercise. Journal of Applied Physiology. 64: 115-119, 1988.

46. Gollnick, P. D., L. A. Bertocci, T. B. Kelso, E. H. Witt, and D. R. Hodgson. The effect of high intensity exercise on the respiratory capacity of skeletal muscle. Pflügers Archives. 415: 407-413, 1990.

47. Gollnick, P. D. and D. W. King. Effect of exercise and training on mitochondria of rat skeletal muscle. American Journal of Physiology. 216: 1502-1509, 1969.

48. Green, M. J. and H. A. O. Hill. Chemistry of dioxygen. Methods in Enzymology. 105: 3-35, 1984.

49. Green, R. C., C. Little, and P. J. O'Brien. The inactivation of isocitrate dehydrogenase by a lipid peroxide. Archives in Biochemistry and Biophysics. 149: 508-605, 1971.

50. Gutteridge, J. M. C. and B. Halliwell. The antioxidant proteins of extracellular fluids. In: Cellular Antioxidant Defense Systems. Ed. by C. K. Chow. Boca Raton, FL: CRC Press, 1988, vol. II, pp. 1-23.

51. Gutteridge, J. M. C. and G. J. Quinlan. Malondialdehyde formation in the thiobarbituric acid test: the role of lipid radicals, iron salts, and metal chelators. Journal of Applied Biochemistry. 5: 293-299, 1983.

52. Halliwell, B. and J. M. C. Gutteridge. Oxygen toxicity, oxygen radicals, transition metals and disease. Biochemical Journal. 219: 1-14, 1984.

53. Harman, D. Free radical theory of aging: consequences of mitochondrial aging. Age. 6: 86-94, 1983.

54. Hellsten, Y., G. Ahlborg, M. Jensen-Urstad, and B. Sjödin. Indication of *in vivo* xanthine oxidase activity in human skeletal muscle during exercise. Acta Physiologica Scandinavica. 134: 159-160, 1988.

55. Hellsten Westing, Y., B. Ekblom, and B. Sjödin. The metabolic relation between hypoxanthine and uric acid in man following maximal short-distance running. Acta Physiologica Scandinavica. 137: 341-345, 1989.

56. Higuchi, M., L-J. Cartier, M. Chen, and J. O. Holloszy. Superoxide dismutase and catalase in skeletal muscle: adaptive response to exercise. Journal of Gerontology. 40: 281-286, 1985.

57. Hill, H. A. O. The chemistry of dioxygen and its reduction products. Ciba Foundation Symposium. 65: 5-17, 1979.

58. Jackson, M. J., R. H. T. Edwards, and M. C. R. Symons. Electron spin resonance studies of intact mammalian skeletal muscle. Biochimica et Biophysica Acta. 847: 185-190, 1985.

59. Jacques, P. F., L. T. Chylack, R. B. McGrandy, and S. C. Hartz. Antioxidant status in persons with and without senile cataract. Archives of Opthamology. 106: 337-340, 1988.

60. Jamieson, J. Oxygen toxicity and reactive oxygen metabolites in mammals. Free Radical Biology and Medicine. 7: 87-108, 1989

61. Jarasch, E. D., G. Bruder, and H. W. Heid. Significance of xanthine oxidase in capillary endothelial cells. Acta Physiologica Scandinavica 126 (Suppl. 548): 39-46, 1986.

62. Jarasch, E. D., C. Grund, G. Bruder, H. W. Heid, T. W. Keenan, and W. W. Franke. Localization of xanthine oxidase in mammary gland epithelium and capillary endothelium. Cell. 25: 67-82, 1981.

63. Jenkins, R. R. The role of superoxide dismutase and catalase in muscle fatigue. In Biochemistry of Exercise. Ed. by H. G. Knuttgen, J. A. Vogel, and J. Pootmans. v. 13, pp. 467-471, 1983.

64. Jenkins, R. R. Free radical chemistry: relationship to exercise. Sports Medicine. 5: 156-170, 1988.

65. Jenkins, R. R., D. Martin, and E. Goldberg. Lipid peroxidation in skeletal muscle during atrophy and acute exercise. Medicine and Science in Sports and Exercise. 15: 93H, 1983.

66. Ji, L. L., D. Dillon and E. Wu. Alteration of antioxidant enzymes with aging in rat skeletal muscle. American Journal of Physiology. 256: R918-923, 1990.

67. Ji, L. L., F. W. Stratman, and H. A. Lardy. Antioxidant enzyme systems in rat liver and skeletal muscle. Archives of Biochemistry and Biophysics. 263: 150-160, 1988.

68. Ji, L. L., F. W. Stratman, and H. A. Lardy. Enzymatic downregulation with exercise in rat skeletal muscle. Archives of Biochemistry and Biophysics. 263: 137-149, 1988.

69. Ketai, L. H., R. H. Simon, J. W. Kreit, and C. M. Grum. Plasma hypoxanthine and exercise. American Review of Respiratory Disease. 136: 98-101, 1988.

70. Korthuis, R. J., D. N. Granger, M. I. Townsley, and A. K. Taylor. The role of oxygen-derived free radicals in ischemia-induced increases in canine skeletal muscle vascular permeability. Circulation Research. 57: 599-609, 1985.

71. Kosaka, H., M. Uozumi, and I. Tyuma. The interaction between nitrogen oxides and hemoglobin and endothelium-derived relaxing factor. Free Radical Biology and Medicine. 7: 653-658, 1989.

72. Lang, J. H., K. Gohil, L. Packer and R. F. Burk. Selenium deficiency, endurance capacity, and antioxidant status in rats. Journal of Applied Physiology. 63: 2532-2535, 1987.

73. Latshaw, S. P., S. Bazaes, A. Randolph, R. A. Poorman, R. L. Heinrikson, and R. G. Kemp. Identification of highly reactive cysteinyl and methionyl residues of rabbit muscle phosphofructokinase. Journal of Biological Chemistry. 262: 10672-10677, 1987.

74. Laughlin, M. H. and R. B. Armstrong. Rat muscle blood flows as a function of time during prolonged slow treadmill exercise. American Journal of Physiology. 244: H814-H824, 1983.

75. Laughlin, M. H. and R. J. Korthuis. Control of muscle blood flow during sustained physiological exercise. Canadian Journal of Sport Science. 12(Suppl. 1): 77S-83S, 1987.

76. Laughlin, M. H., T. Simpson, W. L. Sexton, O. R. Brown, and R. J. Korthuis. Skeletal muscle oxidative capacity, oxidant enzymes, and exercise training. Journal of Applied Physiology. 68: 2337-2343, 1990.

77. Lawler, J., D. Criswell, and S. Powers. Method for disruption of mitochondria in rat diaphragm. Medicine and Science in Sports and Exercise. (Abstract). 21: S70, 1989.

78. Lew, H., S. Pyke, and A. Quintanilha. Changes in the glutathione status of plasma, liver and muscle following exhaustive exercise in rats. FEBS Letters. 185: 262-266, 1985.

79. Mailer, K. Superoxide radical as electron donor for oxidative phophorylation of ADP. Biochemical and Biophysical Research Communication. 170: 59-54, 1990.

80. Marklund, S. L. Ceruloplasmin, extracellular-superoxide dismutase, and scavenging of superoxide anion radicals. Journal of Free Radical Biology and Medicine. 2: 225, 1986.

81. Marklund, S. and G. Marklund. Involvement of the superoxide radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. European Journal of Biochemistry. 47: 469-474, 1974.

82. Maughan, R. J., A. E. Donnelly, M. Gleeson, P. H. Whiting, K. A. Walker, and P. J. Clough. Delayed-onset muscle damage and lipid peroxidation in man after a downhill run. Muscle and Nerve. 12: 332-336, 1989.

83. McCutchan, H. J., J. R. Schwappach, E. G. Enquist, D. L. Walden, L. S. Terada, O. K. Reiss, J. A. Leff, and J. E. Repine. Xanthine oxidase-derived H_2O_2 contributes to reperfusion injury of ischemic skeletal muscle. American Journal of Physiology. 258: H1415-H1419, 1990.

84. Moraski, N., H. Sprecher, G. E. Milo, and D. G. Cornwall. Fatty acid specificity in the inhibition of cell proliferation and its relationship to lipid peroxidation and prostaglandin biosynthesis. Lipids. 17: 893-899, 1982.

85. Nohl, H., D. Hegner, and K.-H. Summer. The mechanism of toxic action of hyperbaric oxygenation on the mitochondria of rat heart cells. Biochemical Pharmacology. 30: 1753-1757, 1981.

86. Novelli, G. P., G. Bracciotti, and S. Falsini. Spin trappers and Vitamin E prolong endurance to muscle fatigue in mice. Free Radical Biology and Medicine. 8: 8-13, 1990.

87. Oberley, L. W., T. O. Oberley, and G. R. Buettner. Cell division in normal and transformed cells. The possible role of superoxide and hydrogen peroxide. Medical Hypothesis. 7: 21-42, 1981.

88. Ohkawa, H., N. Ohishi, and K. Yagi. Assay for lipid peroxidation in animal tissues by thiobarbituric acid reaction. Analytical Biochemistry. 95: 371-379, 1979.

89. Pacifici, R. E. and K. J. A. Davies. Protein degradation as an index of oxidative stress. Methods in Enzymology. 186: 485-500, 1990.

90. Parks, D. A., T. K. Williams and J. S. Beckman. Conversion of xanthine dehydrogenase to oxidase in ischemic rat intestine; a reevaluation. American Journal of Physiology. 245: G285-G289, 1983.

91. Powers, S. K., J. Lawler, D. Criswell, S. Dodd, S. Grinton, G. Bagby, and H. Silverman. Endurance-training-induced cellular adaptations in respiratory muscles. Journal of Applied Physiology. 68: 2114-2118, 1990.

92. Powers, S., J. Lawler, D. Criswell, F. Lieu, and D. Martin. Aging and respiratory muscle plasticity: Effects of endurance training. In Review.

93. Pryor, W. A. Free radical biology: xenobiotics, cancer and aging. Annals of the New York Academy of Sciences. 16: 969-985, 1984.

94. Pryor, W. A. Oxy-radicals and related species. Annual Review of Physiology. 48: 657-667, 1986.

95. Quintanilha, A. T. Effects of physical exercise and/or Vitamin E on tissue oxidative metabolism. Biochemical Society Transactions. 12: 403-404, 1984.

96. Raytch, R. E., R. S. Chuknyiska, and G. B. Bulkley. The primary localization of free radical generation after anoxia/reoxygenation in isolated endothelial cells. Surgery. 102: 122-131, 1987.

97. Reichsteiner, M., S. Rogers, and K. Rote. Protein structure and intracellular stability. Trends in Biochemical Sciences. 12: 390-394, 1987.

98. Rothstein, M. Altered proteins, errors and aging. In Protein Metabolism and Aging Ed. by H. L. Segal, M. Rothstein, and E. Bergamini. New York: Wiley-Liss, 1990, pp. 3-14.

99. Roy, R. S. and J. M. McCord. Superoxide and ischemia: conversion of xanthine dehydrogenase to xanthine oxidase. In: Oxy Radicals and their Scavenger Systems: Cellular and Medical Aspects, Ed. by R. A. Greenwald and G. Cohen. New York: Elsevier/ North Holland Biomedical, 1983, vol. II, pp. 143-153.

100. Sahlin, K. and Broberg, S. Adenine nucleotide depletion in human muscle during exercise: causality and significance of AMP deamination. International Journal of Sports Medicine. 11 (suppl. 2): S62-S67, 1990.

101. Salminen, A. and V. Vihko. Endurance training reduces the susceptibility of mouse skeletal muscle to lipid peroxidation in vitro. Acta Physiologica Scandinavica. 117: 109-113, 1983.

102. Salminen, A. and V. Vihko. Lipid peroxidation in exercise myopathy. Experimental and Molecular Pathology. 38: 380-388, 1983.

103. Scott, J. A. and C. A. Rabitto. Oxygen radicals and plasma membrane potential. Free Radical Biology and Medicine. 5: 237-246, 1988.

104. Sevanian, A. Lipid peroxidation, membrane damage, and phospholipase A_2 action. In: Cellular Antioxidant Defense Systems. Ed. by C. K. Chow. Boca Raton, FL: CRC Press, 1988, vol. II, pp.78-95.

105. Shamsadeen, N. and C. J. Duncan. Cytotoxic effects of oxygen on the skeletal muscle of mouse diaphragm. Virchows Archives B Cell Pathology. 57: 123-129, 1989.

106. Shikimi, T. and S. Masumura. Age and exercise-related changes in the activities of thiol proteinases in rat skeletal muscle. Chemical and Pharmacological Bulletin. 36: 4182-4186, 1988.

107. Shindoh, C., A. DiMarco, A. Thomas, P. Manubag, and G. Supinski. Effect of N-acetylcysteine on diaphragm fatigue. Journal of Applied Physiology. 68: 2107-2113, 1990.

108. Shonk, C. E. and G. E. Boxer. Enzyme patterns in human tissues. I. Methods for the determination of glycolytic enzymes. Cancer Research. 24: 709-721, 1964.

109. Singer, T. Determination of the activity of succinate, NADPH, choline, and alpha-glycerophosphate dehydrogenases. Methods in Biochemical Analysis. 22: 123-175, 1974.

110. Sjödin, B., Y. H. Westing, and F. S. Apple. Biochemical mechanisms for oxygen free radical formation during exercise. Sports Medicine. 10: 236-254, 1990.

111. Slater, T. F., K. H. Cheeseman, M. J. Davies, K. Proudfoot, and W. Yin. Free radical mechanisms in relation to tissue injury. Proceedings of the Nutrition Society. 46: 1-12, 1987.

112. Smith, J. K., D. L. Carden, and R. J. Korthuis. Role of xanthine oxidase in postischemic microvascular injury in skeletal muscle. American Journal of Physiology. 257: H1782-H1789, 1989.

113. Smith, J. K., M. B. Grisham, D. N. Granger, and R. J. Korthuis. Free radical defense mechanisms and neutrophil infiltration in postischemic skeletal muscle. American Journal of Physiology. 256: H789-H793, 1989.

114. Soussi, B., J.-P. Idström, T. Scherstén, and A.-C. Bylund-Fellenius. Cytochrome c oxidase and cardiolipin alterations in response to skeletal muscle ischaemia and reperfusion. Acta Physiologica Scandinavica. 138:107-114, 1990.

115. Southern, P. A. and G. Powis. Free radicals in medicine. I. Chemical nature and biological reactions. Mayo Clinic Proceedings. 63: 381-389, 1989.

116. Srere, P. Citrate synthase. Methods in Enzymology. 13: 3-11, 1969.

117. Starnes, J. W., G. Canter, R. P. Farrar, and J. P. Kehrer. Skeletal muscle lipid peroxidation in exercised and food restricted rats during aging. Journal of Applied Physiology. 67: 69-75, 1989.

118. Stocks, J., J. M. L. Gutteridge, R. J. Sharp, and T. L. Dormandy. The inhibition of lipid peroxidation by human serum and its relation to serum proteins and œ-tocopherol. Clinical Science and Molecular Medicine. 47: 223-233, 1974.

119. Tani, M. Effects of anti-free radical agents on Na^+, Ca^{2+}, and function in reperfused rat hearts. American Journal of Physiology. 259: H137-H143, 1990.

120. Terjung, R. L., K. M. Baldwin, P. A. Molé, G. H. Klinkerfuss, and J. O. Holloszy. Effect of running to exhaustion on skeletal muscle mitochondria: a biochemical study. American Journal of Physiology. 223: 549-554, 1972.

121. Tolmasoff, J. M., T. Ono, and R. G. Cutler. Superoxide dismutase: correlation with life-span and specific metabolic rate in primate species. Proceedings of the National Academy of Sciences USA. 77: 2777-2781, 1980.

122. Tullson, P. C. and R. L. Terjung. Adenine nucleotide degradation in striated muscle. International Journal of Sports Medicine. 11 (suppl. 2): S47-S55, 1990.

123. Ursini, F. The phospholipid hydroperoxide glutathione peroxidase: a peroxidation inhibition enzyme. In: Cellular Antioxidant Defense Systems. Ed. by C. K. Chow. Boca Raton, FL: CRC Press, 1988, vol. II, pp. 69-76.

124. Wayner, D. D. M., G. W. Burton, K. U. Ingold, and S. Locke. Quantitative measurement of the total peroxyl radical-trapping antioxidant capability of human blood plasma by controlled peroxidation. The important contribution made by plasma proteins. FEBS Letters. 187: 33, 1987.

125. Wittenberg, B. A. and J. B. Wittenberg. Transport of oxygen in muscle. Annual Review of Physiology. 51: 857-878, 1989.

126. Wu, E., L. L. Ji, M. Meyers, and D. P. Thomas. Training improves metabolic and antioxidant functions in skeletal muscle of the aged rat. Medicine and Science in Sports and Exercise. (Abstract). 21: S16, 1989.

127. Yokota, J., J. P. Minei, G. A. Fantini, and G. T. Shires. Role of leukocytes in reperfusion of skeletal muscle following partial ischemia. American Journal of Physiology. 257: H1068-H1075, 1989.

128. Zerba, E., T. E. Komorowski, and J. A. Faulkner. Free radical injury to skeletal muscles of young, adult, and old mice. American Journal of Physiology. 258: C429-C435, 1990.

129. Zhang, L., M. Maiorino, A. Roveri, and F. Ursini. Phospholipid hydroperoxide glutathione peroxidase specific activity in tissues of rats of different ages and comparisons

with other glutathione peroxidases. Biochimica et Biophysica Acta. 1006: 140-143, 1989.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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